University of Alberta

# *In vivo* and *In vitro* Studies of Anticancer Defense: Diet, Exercise and Immunity

by

Lindsay Elaine Robinson Parsons ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

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# ABSTRACT

This study investigated the role of diet and exercise in anticancer immune defense. Feeding a physiological level of long-chain n-3 fatty acids altered membrane lipid composition of resting and activated immune cells and enhanced immunity in healthy rats. Furthermore, although long-chain n-3 fatty acids and exercise were immunostimulatory on an individual basis, their combination did not enhance immunity. A second objective was to examine the anticancer effects of long-chain n-3 fatty acids fed in either a high or low polyunsaturated/saturated fat (P/S) diet using an animal model of cancer (R3230AC mammary adenocarcinoma). Although tumour growth was not significantly affected by diet, long-chain n-3 fatty acids were more effective at altering tumour cell membrane composition and improving anticancer immunity when supplemented in a low P/S diet. In an *in vitro* study using the MDA-MB-231 cell line, incubation with the long-chain n-3 fatty acid, docosahexaenoic acid (DHA) (with or without linoleic acid) altered tumour membrane composition, decreased tumour cell proliferation, but did not affect apoptosis. The mechanism for DHA-induced growth inhibition was not alteration in cell cycle phase distribution or in expression of D-type cyclins or proliferation-related proteins (PCNA and PRK). However, DHA (with linoleic acid) significantly decreased cyclin B1 expression in S phase and increased tumour suppressor proteins (p53 and pRb). A final objective was to study the role of diets supplemented with glutamine, arginine, or ornithine α-ketoglutarate (OKG) in modulating antitumour immune function in rats. Even though tumour growth was not inhibited by amino acids, dietary OKG was more effective than glutamine or arginine at enhancing host immune function. In summary, a number of effects of diet and exercise on anticancer immune defense were studied and the mechanisms for those effects investigated. Although tumour growth was not significantly inhibited by diet, the ability of specific nutrients (e.g. long chain n-3 fatty acids, OKG) to upregulate the immune system may be beneficial in aspects other than primary tumour growth inhibition, such as long-term cancer prevention or metastasis. Therefore, this research provides rationale for recommendations as to dietary fat composition, amino acid supplementation and exercise-training optimal for anticancer immune defense.

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## Faculty of Graduate Studies and Research

The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled ***In vivo* and *In vitro* Studies of Anticancer Defense: Diet, Exercise and Immunity** submitted by Lindsay Elaine Robinson Parsons in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Nutrition and Metabolism.

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This work is dedicated to my parents, Paul and Elaine Robinson,
for their ongoing interest in my scientific endeavours
and
to my husband, Glenn Parsons,
for his continual belief in my scientific abilities

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# LIST OF ABBREVIATIONS

AA = arachidonic acid

ANSA = 8-anilino-1-naphthalene sulfonic acid

AOAC = Association of Official Analytical Chemists

ANOVA = analysis of variance

AOAC = Association of Official Analytical Chemists

APC = antigen-presenting cell

Arg = arginine

BSA = bovine serum albumin

°C = degrees Celsius

CCM = complete culture media

Cdk = cyclin dependent kinase

Con A = concanavalin A

CTL = cytotoxic T lymphocyte

DHA = docosahexaenoic acid

DMBA = dimethylbenz(a)anthracene

DNA = deoxyribonucleic acid

DPA = docosapentaenoic acid

ELISA = enzyme linked immunosorbent assay

EPA = eicosapentaenoic acid

FCS = fetal calf serum

FITC = fluorescein isothiocyanate

Gln = glutamine

HEPES = hydroxyethylpiperazine-N'-2-ethanesulfonic acid

HPLC = high performance liquid chromatography

IFN-γ = interferon-gamma

Ig = immunoglobulin

IL = interleukin

KRH = Krebs' Ringer HEPES

LA = linoleic acid

LNA = α-linolenic acid

LPS = lipopolysaccharide

mAb = monoclonal antibody

MH = Morris hepatoma

MHC = major histocompatability complex

MUFA = monounsaturated fatty acid

NK = natural killer

NMU = N-methyl-N-nitrosourea

NO = nitric oxide

$NO_2^-$ = nitrite

OKG = ornithine α-ketoglutarate

Orn = ornithine

PBS = phosphate buffered saline

PC = phosphatidylcholine

PCNA = proliferating cell nuclear antigen

PE = phosphatidylethanolamine

PI = phosphatidylinositol

PMA = phorbol myristate acetate

PRK = proliferation-related kinase

P/S ratio = polyunsaturated/saturated fatty acid ratio

PS = phosphatidylserine

PUFA = polyunsaturated fatty acid

Rb = retinoblastoma

RIPA = radioimmunoprecipitation assay

SAS = statistical analysis system

SEM = standard error of the mean

SFA = saturated fatty acid

SM = sphingomyelin

TAA = tumour-associated antigen

TBS = tris buffer saline

TCR = T cell receptor

Th = T helper cell

TLC = thin layer chromatography

TNF = tumour necrosis factor

TPN = total parenteral nutrition

U.I. = unsaturation index

# 1. INTRODUCTION AND LITERATURE REVIEW

## 1.1 CANCER

It is currently estimated that approximately 1 in 3 Canadians will develop some form of cancer (excluding non-melanoma skin cancer) during their life (National Cancer Institute of Canada 2000). Cancer is not a single disease but rather the generic name used for more than 100 distinct types of the disease, all characterized by uncontrolled cell growth (Hanahan and Weinberg 2000). While normal cells divide and grow in an orderly manner, cancer cells do not, resulting in disruption of normal body tissues and organ functions (Hanahan and Weinberg 2000). In general, tumours are derived from only one or a few normal cells which have defects in regulatory mechanisms that govern normal cell proliferation and homeostasis (Hanahan and Weinberg 2000). Malignant tumours then grow in an uncontrolled manner, invade normal tissues, and often metastasize and grow at sites distant from the tissue of origin (Weinberg 1996a). The next sections of this literature review will focus primarily on breast cancer.

Recent Canadian cancer statistics estimate that approximately 1 in 9 Canadian women will develop breast cancer during her lifetime (National Cancer Institute of Canada 2000). In fact, breast cancer is the most frequently diagnosed cancer (excluding non-melanoma skin cancer) in Canadian women, accounting for about 30% of new cancer cases each year (National Cancer Institute of Canada 2000).

### 1.1.1 Cell Cycle

In eukaryotes, cell growth and division occur via a sequence of physiological events referred to as the cell cycle (Nurse 2000). Cell cycle control is the major regulatory mechanism of cell growth and development of all living organisms (Prescott 1976). Cells may be either actively proliferating or in a quiescent state ($G_0$) in which they have withdrawn from the cell cycle (Prescott 1976). The cell cycle can be subdivided into four phases: $G_1$, S, $G_2$, and M (Prescott 1976). S-phase (DNA synthesis phase) is the period during which the genome is replicated in preparation for division and M-phase (mitosis) is the period during which cell division occurs (Prescott 1976). The $G_1$ and $G_2$ phases follow upon mitosis and S-phase, respectively (Prescott 1976).

Increasing genetic instability during tumour progression creates malignant cells with disorganization of various functional systems, including the cell cycle. Thus, a characteristic feature of most, if not all, malignant tumours is deregulation of the cell cycle, which can occur on many levels, including one or several of the proteins involved in cell cycle control (Landberg and Roos 1997).

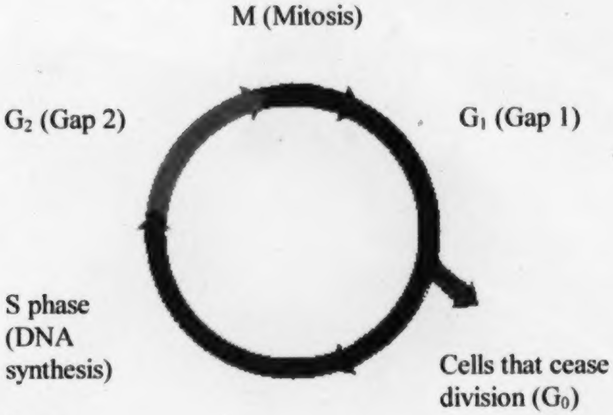

FIGURE 1.1 Cell Cycle

### 1.1.2 Cell Cycle Machinery

The cell cycle machinery which regulates cell proliferation is composed of two principal components, cyclin dependent kinases (cdks) and cyclins (Nurse 2000). The cdks are present throughout the cell cycle, but become active only when they interact with their cyclin partners (Nurse 2000). As their name signifies, cyclin proteins are synthesized and degraded in an orderly, scheduled manner within the normal, noncancerous cell (Nurse 2000). Levels of particular cyclins are regulated at the transcription level, as well as by targeted degradation via the ubiquitin pathway (Murray 1995). A variety of cyclin:cdk complexes are formed during distinct phases of the cell cycle, each dedicated to the phosphorylation of a distinct set of target proteins (Lundberg and Weinberg 1999).

Simplified, cyclins can be divided into $G_1$ cyclins and mitotic cyclins, which are involved in the $G_1$-S and $G_2$-M transition, respectively (Koff et al. 1991). For example, D- and E-type cyclins are expressed during $G_0/G_1$ and are referred to as start cyclins. Start, also known as the restriction checkpoint (R-point) in mammalian cells, is the point in late $G_1$ when the cell commits itself to another round of DNA replication. The D-type cyclins (D1, D2, D3) appear to promote $G_0$ to $G_1$ transitions and the rate of $G_1$ progression. The D-type cyclins are expressed in response to growth factors or mitogens, and rapidly degrade when mitogens are withdrawn. Once synthesized, the D-type cyclins associate with cdk4 and cdk6, two alternative partner cdks whose functional distinctions have yet to be elucidated (Lundberg and Weinberg 1999). In general, cyclin E is induced later in $G_1$ (several hours before the onset of S phase) than the D-type cyclins and forms complexes with cdk2 (Koff et al 1991). The activity of this complex appears to be essential for entrance into S phase. Cyclins A and B1 are called mitotic cyclins. Specifically, Cyclin A is synthesized during S phase and degrades during anaphase, while Cyclin B1 is synthesized during late S, maximally expressed during the $G_2$ to M transition, and degraded during anaphase (Pines 1995) Initially, cyclin A associates with cdk2 and later with cdc2. This association continues until the appearance of the B-type cyclins which form complexes with cdc2, triggering the events associated with mitosis (Pines 1995). Overall, the activities of cdks, which are manifested periodically at specific times of the cell cycle, affect all of the major transitions of the cell cycle (Lundberg and Weinberg 1999). To ensure that growth and division through mitosis (M phase) is properly completed, a series of checkpoint controls operate during the remainder of the cell cycle (Lundberg and Weinberg 1999). For example, checkpoint controls ensure that S phase DNA replication is completed before preparations for M phase entrance are undertaken; that genomic damage stops cell cycle progression until the genome is made complete once again; and that advance through M phase is stopped until disrupted spindles become properly assembled and aligned (Nurse 2000).

Although cdks are responsible for triggering the events which drive cell cycle progression, other types of molecules are involved in restricting or facilitating the action of these enzymes. For example, the stimulation of cells in $G_0$ to proliferate requires the presence of growth factors and the transmission of their proliferative signals from cell surface receptors to the nucleus, where the transcription program of the cell is affected. Some of the proteins expressed as a result of this process, such as c-Fos, c-Jun, and c-Myc, are believed to help set the cell cycle in motion and allow the cell to proceed through early $G_1$ (Hofbauer and Denhardt 1991).

In contrast to gene products which promote cell proliferation, other proteins are involved in arresting it. For example, a number of cdk inhibitors have been discovered which play roles in arresting cell cycle progression by directly binding to and interfering with the activity of cdks (Elledge and Harper 1994). Some of the inhibitors, such as p16, inhibit specific cdk-complexes, whereas others can inhibit several cdks (Biggs and Kraft 1995) Other proteins, such as the *retinoblastoma* (*Rb*) gene product, pRb, which is present in normal cycling mammalian cells, serve as part of a braking mechanism acting on the cell cycle. The phosphorylation state of pRb impacts upon the capacity of the cell to proceed into S phase (Hollingsworth et al. 1993). In $G_1$, D-type cyclins bind to cdks 4 or 6 and the resulting complexes act on pRb, releasing the braking effect of pRb and enabling the cell to progress into late $G_1$ and then into S phase (Sherr 1993). The signal transduction events leading up to pRB phosphorylation and/or functional inactivation are disrupted in many and perhaps all types of cancer cells (Hatakeyama and Weinberg 1995).

Other molecules function in restraining cell cycle progression in extraordinary circumstances, as elements of checkpoint controls in response to physiological problems in the cell. For example, the *p53* gene encodes a nuclear phosphoprotein, which normally functions in the context of DNA damage as a cell cycle checkpoint regulator to slow cell growth and induce DNA repair (Kastan et al. 1991). At a molecular level, p53 induces growth arrest in multiple points of the cell cycle through induction of a cdk inhibitor, p21, (alternatively known as WAF1, Cip1 and Cap20), which ultimately arrests the cell cycle (el-Deiry et al. 1993). Levels of p53 have been shown to increase within 30 min of DNA damage (Kastan et al 1991). If damage is too severe for repair, cell death (apoptosis) is triggered. Thus, p53 acts as a master governor of two distinct downstream machineries: it may inhibit cell cycle progression or may induce apoptosis. During normal physiological circumstances, p53 is present only at low levels, and is induced specifically as part of a checkpoint response to loss of genomic integrity (Lane et al. 1995). In general, mutation of *p53* is associated with enhanced genetic instability in cancer (Kirsch and Kastan 1998).

### 1.1.3 Cell Cycle and Cancer

Properly functioning cell cycle machinery and cell cycle regulators are critical to normal cell growth and division. Multiple genetic changes are necessary in order to transform a cell and alterations in checkpoints surrounding DNA replication and mitosis might decrease the accuracy of the replication, leading to further genetic damage (Landberg and Roos 1997). In recent years, there has been an increased focus on connections between cell cycle control and cancer development (Hartwell and Kastan 1994). For example, overactivity of the D-type cyclins, cyclin E and cdk 4 have been implicated in certain human cancers (Pines 1995). As well, in breast cancer cells, there is often an overabundance of D-type cyclins and cyclin E (Pines 1995). Unscheduled expression of $G_1$ cyclins during $G_2$/M, and vice versa, suggests that their partner cdks may remain persistently active throughout the cell cycle (Darzynkiewicz et al. 1996). This may result in a loss of the regulatory control mechanisms at particular checkpoints of the cycle. The evidence of unscheduled expression of cyclins may be of prognostic value in oncology.

During the progression of breast cancer from initiation to metastasis, mutation, inactivation, loss, or down-regulated expression of tumour-suppressor genes is common (Lippman et al. 1986). For example, the *retinoblastoma* and *p53* genes are mutated in approximately 20 and 50% of cases, respectively (Allred et al. 1994). The *p53* tumour suppressor gene is the most frequently mutated gene yet identified in human cancer (Levine 1997). Abnormalities in *p53* affect genomic instability and appear to facilitate amplification of the *erbB2* oncogene, thought to be a growth factor receptor and associated with poorer prognosis (Eyfjord et al. 1995). Theoretically, any participants in cell cycle control can be deregulated in tumours, however, inactivation of pRb likely represents the ultimate defect in the machinery controlling the $G_1$-S transition (Lukas et al. 1995). The net effect of any of these changes is deregulation of the cell cycle and, in turn, excessive cell proliferation (Weinberg 1996b). Owing to the proposed abundance of cell cycle alterations in tumours, cell cycle defects might even be obligatory in cancer development (Strauss et al. 1995).

### 1.1.4 Apoptosis

The growth of a tumour is determined by the balance between cell proliferation and cell loss (Lundberg and Weinberg 1999). Cell loss occurs by either necrosis or apoptosis. Necrosis, which may occur in response to injury by toxins, physical stimuli, or ischemia, is characterized by cell swelling, disruption of membranes and lysis of the nuclear chromatin (Saikumar et al. 1999). Apoptosis, or programmed cell death, is a biochemical process in which cells are programmed to die under a range of physiological and developmental factors (Saikumar et al 1999). Apoptosis is one of the most important events governing the behaviour and fate of cells in the body and is one of the central mechanisms in tumourigenesis. Cells in the process of apoptosis undergo serial changes, including increased intracellular calcium, membrane blebbing, morphological changes and DNA fragmentation which eventually lead to cell death and formation of apoptotic bodies (Saikumar et al 1999). In contrast to necrosis, membrane damage occurs late during apoptosis, and dead cells are phagocytosed, resulting in little or no inflammatory response.

There are many intracellular mediators which modulate apoptosis, such as cell cycle regulators, p53, the Bcl family of proteins, and Fas-associated death domain. In cancer cells, the lack of an appropriate signal or a defect in the mechanisms leading to induction of apoptosis results in cells that become deregulated in their growth control and less apoptotic (Saikumar et al 1999). Genes that have received the most attention for their roles in apoptosis include *Bcl-2* and *p53* (Engel et al. 1998). Transpositions at the Bcl-2 locus result in overexpression of the Bcl-2 protein, decreased death of lymphoid cells, and B cell lymphoma (Korsmeyer 1992). As discussed above, p53 is a transcription factor that induces cell cycle arrest if DNA damage is too severe for repair (Schwartz and Rotter 1998). Mutations or deletions of *p53* compromise the normal functions of the protein and allow cells to survive under conditions when they should be deleted (Amundson et al. 1998). Methods of promoting cancer cell apoptosis may have potential in cancer prevention and treatment.

### 1.1.5 Overview of Risk Factors

Inherited mutations of the genes *BRCA-1*, *BRCA-2*, and the *Ataxia Telangectasia* gene (*ATM*) suggest a very high risk of breast cancer for the small number of women affected (Bieche and Lidereau 1995). Specifically, such mutations account for only about 5% of all breast cancers (Easton 1994, Eeles et al. 1994). Age is the most significant risk factor for breast cancer (Bryant and Brasher 1994). Other risk factors include a family history of breast cancer, a history of the disease in one breast (or of certain types of benign breast disease), and high levels of radiation exposure to the chest (Kelsey and Bernstein 1996). Obesity in post-menopausal women and various reproductive risk factors, such as never having had children (nulliparity), late age at first full-term pregnancy, early onset of menarche, and late onset of natural menopause are also well-established risk factors (Kelsey and Bernstein 1996). In general, longer reproductive lifetime that includes later and fewer births results in higher breast cancer rates. These observations have implicated endogenous hormones, particularly estrogens, as underlying biological determinants of breast cancer incidence (Pike et al. 1983). Demographic factors that increase breast cancer risk include living in an urban area, belonging to a higher socioeconomic class, and being born in North America or Northern Europe (Kelsey and Bernstein 1996). While most of these aforementioned risk factors cannot be modified, other lifestyle-related risk factors are more amenable to modification. For example, there is increasing evidence linking physical inactivity and dietary factors, such as alcohol use and a high fat intake to the risk of breast cancer (Kelsey and Bernstein 1996). The World Cancer Research Fund estimates that 60-70% of cancers, as well as 35% of all cancer deaths, may be related to lifestyle factors such as diet (World Cancer Research Fund 1997). One critical lifestyle factor, dietary fat, will be discussed in more detail.

## 1.2 DIETARY FAT

The omega-3 (n-3) and omega-6 (n-6) polyunsaturated fatty acids (PUFA) are referred to as essential fatty acids because they cannot be synthesized by mammals and must be obtained from the diet (Spector 1999). Specifically, the essential n-3 and n-6 fatty acids are α-linolenic (18:3n-3, LNA) and linoleic acids (18:2n-6, LA), respectively. Both

LNA and LA are metabolized to longer-chain fatty acids, primarily in the liver. Specifically, LNA is converted to eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), while LA is metabolized to arachidonic acid (20:4n-6, AA) (Sprecher 1989). These metabolic events involve increases in chain length and degree of unsaturation that are achieved by adding extra double bonds between the existing double bond and the carboxyl group (de Gommez Dumm and Brenner 1975). There is competition between the n-3 and the n-6 fatty acids for the Δ4 and Δ6 desaturases, with the n-3 fatty acids having greater affinities for the enzymes. As well, increasing the dietary intake of n-3 fatty acids reduces the desaturation of LA and thus, AA production (Garg et al. 1990). This inhibition of AA is achieved by EPA and DHA, as well as LNA (Garg et al 1990). Humans can produce EPA from LNA, but the extent of the capacity to perform this conversion has not been well defined (Hunter 1990). Retroconversion of DHA to EPA also occurs in humans (Conquer and Holub 1997).

### 1.2.1 Dietary Fat and Breast Cancer

There is a long debated relationship between the incidence of cancer and diet, especially dietary fat (Hunter and Willett 1996, Carroll 1975). Numerous experimental studies provide evidence linking high dietary fat consumption with increased risk of cancer at select sites, notably breast, colon, and prostate (Birt 1990). However, there is increasing evidence that certain types of dietary fat may be anticarcinogenic (Rose 1997, Ip 1997, Cave 1991). This relationship has been well demonstrated in the Inuit population, who have a low cancer incidence, despite consuming a diet rich in fat (Gaudette et al. 1996, Sinclair 1953). In addition, many animal model studies have shown that diets containing high levels of n-3 PUFAs inhibit tumour development and growth (Ip 1997, Welsch 1995, Carroll 1987). The majority of these investigations have fed the marine-oil derived long-chain n-3 fatty acids, notably EPA and DHA. Plant-derived n-3 fatty acids (primarily LNA) have not been as widely studied as those of marine origin in terms of their influence on cancer promotion.

### 1.2.1.1 Epidemiological Evidence

In humans, evidence for a relationship between dietary fat and cancer comes primarily from epidemiologic studies, which often show conflicting results. In general, ecologic or international correlations demonstrate a positive relationship between increased dietary fat intake and increased cancer risk (Carroll and Khor 1975). In addition, migration studies have consistently shown that when females move from a low- to a high-incidence country their breast cancer rates increase within one generation (Ziegler et al. 1993).

Other types of human studies which focus on total dietary fat intake include case-control and prospective (cohort) studies. Case-control studies are based on comparisons of dietary practices between cancer patients and controls by estimating food intake relating to the period prior to clinical manifestation of the disease. In cohort studies, dietary practice is determined among a large number of people and related to subsequent occurrence of cancer. In general, case-control studies have not identified a significant association between total fat intake and risk of breast cancer (Willett 1999). However, a meta-analysis of 12 case-control studies has shown a positive relationship between total dietary fat consumption and breast cancer in postmenopausal women (Howe et al. 1990). In contrast, a pooled analysis of all large cohort studies found no specific relationship between total fat intake and breast cancer risk (Hunter and Willett 1996). There are several limitations to these types of epidemiologic studies which have been discussed in detail elsewhere (Hunter and Willett 1996, Hunter and Willett 1993). For example, the inability to clearly distinguish between the effects of different types of fat comprising total dietary fat intake is one limitation of such studies.

Thus, epidemiologic studies have also attempted to determine whether correlations exist between specific types of dietary fat, such as the long-chain n-3 PUFAs, and cancer. The diets of Canadian Inuit and the aboriginal population in Greenland and Alaska are high in fat, but the source is largely from marine mammals (Bang et al. 1976) and fish (Nobmann et al. 1992). As a result, these populations have very high intakes of n-3 fatty acids, which are reflected in the plasma and erythrocyte membrane EPA and DHA levels (Innis et al. 1988, Bates et al. 1985). Although such epidemiological studies of defined populations support a cancer protective role for dietary n-3 fatty acids, it should be noted that cancer protective factors, such as early age at first pregnancy and prolonged lactation, are also prevelant among the Inuit population which likely contribute to their low breast cancer risk.

A prospective cohort study in northern Sweden found no association between n-3 fatty acids in serum phospholipids (used as biomarkers of previous fatty acid intake) and risk of breast cancer (Chajes et al. 1999). Similar results were found in prospective studies performed in both the United States (Holmes et al. 1999) and in Norway (Vatten 1990), a country with a relatively high consumption of fish-derived n-3 fatty acids. A detailed correlation analysis of international data reported that total PUFA intake is positively correlated with the incidence of breast and prostate cancers, while long-chain n-3 fatty acids from fish (0.04 -1.5 g/d) have a nonsignificant negative association with the cancer types studied, particularly breast cancer (Hursting et al. 1990). However, more recent epidemiologic studies suggest that consumption of a diet rich in long-chain n-3 fatty acid-containing fish reduces breast cancer risk (Simonsen et al. 1998, Sasaki et al. 1993). It should be noted that, in some countries, intake of n-3 fatty acids is relatively small compared with total PUFA intake, a factor which may impact on the ability of n-3 fatty acids to alter cancer risk. This is supported by Dolecek (1992) who reported a protective relationship between the ratio of dietary n-6 to n-3 PUFAs and cancer mortality (Dolecek 1992). Specifically, both the LA/LNA ratio and the total n-6/n-3 fatty acid ratio in the diet had significant inverse relationships with cancer mortality. Thus, this study suggests that the ratio of n-6/n-3 fatty acids in the diet, and not the absolute level of n-3 PUFAs, may be the dietary factor responsible for cancer protection. Recently, Simonsen et al. (1998) reported a case-control study in postmenopausal breast cancer cases and controls, in five European countries differing greatly in dietary fat intakes and breast cancer risk (European Community Multicentre Study on Antioxidants, Myocardial Infarction, and Cancer of the Breast (EURAMIC), 1991-92). In this study, adipose tissue fatty acid composition was used as a biomarker of PUFA intake. Similar to previous studies, they found no significant inverse association between total n-3 fatty acid intake and breast cancer risk. In contrast, there was an inverse association between the ratio of long-chain n-3 fatty acids to total n-6 PUFAs and breast cancer in four of five centers (Simonsen et al 1998). This data strengthens the hypothesis that the breast cancer protective effect of dietary n-3 fatty acids might depend on total n-6 PUFA intake.

Future dietary fat and cancer studies must give more consideration to the dietary n-6/n-3 fatty acid ratio as opposed to the absolute levels of these fatty acids, a factor which has been the focus of many previous studies in this area. Although epidemiologic data may provide support for the suggestion that n-3 PUFAs protect against certain cancer types, they cannot determine the mechanism by which dietary fat affects tumour growth. Thus, additional types of experimental studies are required to study the relationship between dietary fat and cancer.

#### 1.2.1.2 Animal Model Studies

Several animal model studies have attempted to determine whether or not n-6 and n-3 PUFAs have similar effects on tumour growth. In general, studies have shown that significant differences exist between the tumour-enhancing potential of a high fat diet rich in n-6 fatty acids and that of one composed predominantly of n-3 fatty acids. Specifically, it has been shown that rodents fed diets high in n-3 PUFAs exhibit suppression of tumourigenesis when compared to rodents fed high n-6 fatty acid diets (Rose and Connolly 1999, Cave, Jr. 1997, Rose 1997, Ip 1997, Welsch 1995, Karmali 1989, Carroll 1987).

In general, the n-6 fatty acids have been associated with enhancement of the promotional phase of experimental mammary tumourigenesis (Rose 1997). In fact, animal model studies of various cancer types, including breast, have shown a requirement for n-6 fatty acids in the enhancement of tumourigenesis by dietary fat (Carroll et al. 1986). For example, Ip et al. (1985) have determined a requirement for the essential fatty acid LA for mammary tumourigenesis in rats treated with the carcinogen dimethylbenz(a)anthracene (DMBA). Specifically, this study demonstrated that the yield of mammary tumours progressed linearly in proportion to increasing dietary levels of LA from 0.5% to 4% (w/w in diet) (Ip et al. 1985). At amounts above 4% LA (w/w) in the diet tumour development leveled off, suggesting that a maximal response had been reached. Although the mechanism for this positive dose-response relationship between LA and tumour growth remains unclear, it has been concluded that 4% (w/w) is the threshold level of dietary LA required for maximal tumour growth stimulation (Ip et al 1985). It should be noted that the concept of a 4% (w/w) dietary LA requirement in tumourigenesis has not been established in all experimental tumour models and might in fact be highly specific for each organ site. Since it is possible that different tumour models may have a unique response to the availability of LA in the diet, this finding must not be generalized to include all cancer models, including human cancers.

Some of the earliest work involving the long-chain n-3 PUFAs and rat mammary tumourigenesis was done by Jurkowski and Cave (1985) and Braden and Carroll (1986) using the N-methyl-N-nitrosourea (NMU)- and DMBA-induced mammary tumour models, respectively. These studies showed that as the percentage of dietary menhaden (fish) oil (rich in long-chain n-3 fatty acids) increased there was a reduction in tumour development, while corresponding dietary levels of corn oil (rich in n-6 fatty acids) enhanced mammary tumour growth. These studies have attributed the inhibitory effects of fish oil on tumourigenesis to the presence of the long-chain n-3 fatty acids, EPA and DHA, in the diet. Other researchers have also shown that chemically-induced rat mammary carcinogenesis is promoted by dietary n-6 fatty acids, but inhibited by feeding n-3 fatty acid-rich fish oil (Cohen et al. 1993, Abou-el-Ela et al. 1989, Abou-el-Ela et al. 1988). However, the fish oil diets used in these particular investigations were not supplemented with the essential LA and therefore did not have sufficient LA to meet the threshold level required for maximal tumour growth as suggested by Ip et al. (1985) (Welsch 1992). Thus, it is possible, that it is the suboptimal level of LA that is suppressing tumour growth as opposed to the fish oil itself. That is, the long-chain n-3 PUFAs *per se* may not be inhibiting tumour growth in these studies. In fact, a limitation of many previous studies using diets rich in n-3 PUFAs has been that the associated LA levels have been inadequate to meet essential fatty acid requirements for tumour promotion as suggested by Ip et al. (1985).

Compared to the wide array of studies which have looked at the effects of altering dietary fat composition on the growth of carcinogen-induced tumours in animals, there are relatively fewer studies examining the effects of changing dietary fat composition on growth of transplantable mammary tumours, such as the R3230AC adenocarcinoma. In one of the earliest investigations on the effects of n-3 fatty acids on the development of this tumour model, it was demonstrated that daily oral supplementation of a rat chow diet (5% w/w mixed fat) with 17, 33, and 67 mg EPA and 16, 32, and 64 mg DHA provided by a commercial fish oil concentrate (MaxEPA) significantly reduced tumour growth compared with rats fed the chow diet without added long-chain n-3 PUFAs (Karmali et al. 1984). In subsequent experiments using the R3230AC tumour, different combinations of fish oil and corn oil were added to semi-purified diets at 23.5% (w/w) (Karmali et al. 1987). In this study there was only a slight reduction in tumour weight in the group of rats fed the diet containing 11.75% (w/w) MaxEPA + 11.75% (w/w) corn oil (total n-6/n-3 = 1.7) compared to those rats fed the control 23.5% (w/w) corn oil diet. However, in the group of rats fed the diet containing 18.5% MaxEPA + 5% corn oil (total n-6/n-3 = 0.5) tumour weight was reduced approximately 50% compared to the control corn oil-fed animals.

The recent availability of pure EPA and DHA ethyl esters and microalgae-derived DHA-rich oils provides a new approach to n-3 fatty acid chemoprevention and suppression of cancer progression (Rose and Connolly 1999). Furthermore, it is now possible to compare the efficacy of EPA and DHA. For example, Noguchi et al. (1997) used the DMBA-induced mammary tumour model to examine the effects of low doses of EPA and DHA ethyl esters on tumour incidence and growth in rats fed a 20% (w/w) corn oil diet. In this experiment, 0.5 mL of pure n-3 fatty acid was given by gastric gavage twice a week to provide a dietary n-6/n-3 fatty acid ratio of 1.8. Compared with control rats, tumour incidence was reduced 69 and 47% in DHA- and EPA-treated rats, respectively.

In a meta-analysis of 97 rodent studies extending from 1966 to 1994, Fay et al. (1997) showed that n-6 fatty acids have a strong mammary tumour-enhancing effect, saturated fats have a weaker tumour-enhancing effect, and n-3 fatty acids have a small protective effect. However, the ratio of n-3 fatty acids with other types of fatty acids was not considered. Cave (1997) has reviewed certain animal model studies investigating the effects of dietary mixtures of n-6 and n-3 PUFAs on mammary tumourigenesis and has suggested that a low level of dietary n-3 fatty acids may not have a detectable suppressor effect on tumour growth when accompanied by sufficient dietary n-6 fatty acids. However, when the proportion of dietary n-3 fatty acids is much greater than that of n-6 fatty acids in the diet, an inhibitory effect of n-3 fatty acids on tumourigenesis has been observed. It appears, then, that the ratio of n-6 to n-3 fatty acids in the diet may be the critical determinant of the influence of PUFAs on tumour promotion as opposed to the absolute levels of fatty acids in the diet. However, research studies have yet to fully establish the optimal n-6 to n-3 fatty acid balance in the diet which is essential for the tumour inhibitory effect of n-3 fatty acids to be fully expressed. Thus, future studies should place more emphasis on the ratio of dietary n-6 to n-3 fatty acids as opposed to the absolute levels of fatty acids in the diet. Furthermore, there is evidence that when fish oil is fed as 18% (w/w) of the diet, it may actually enhance mammary tumour development (Cohen et al 1993). However, it is questionable whether such an extreme level of fish oil has any implications for the design of human dietary intervention trials, when it would be extremely difficult to make such a substantial change in the habitual dietary intake of individuals in the population.

#### 1.2.1.3 Summary

A recent epidemiologic study by Simonsen et al. (1998) suggests that consumption of a diet rich in fish-derived long-chain n-3 fatty acids reduces breast cancer risk. In laboratory studies, dietary supplementation of fish oil (Ip 1997) or pure n-3 fatty acids (Noguchi et al. 1997) has been shown to suppress tumour development in chemically-induced rat mammary tumour models, inhibit growth of transplantable rat mammary tumours (Karmali et al 1984), and suppress growth and metastasis of human breast cancer cells in athymic nude mice (Rose et al. 1995). However, the precise role of the long-chain n-3 fatty acids, EPA and DHA in the inhibition of mammary tumour growth remains uncertain. In most cases, high amounts of n-3 fatty acids were fed to induce inhibition of tumour growth (Cave, Jr. 1997, Ip 1997, Welsch 1995). However, as discussed above, a number of these studies have attributed tumour inhibition to the presence of EPA and

DHA in fish oil when in fact the suppressor effect may have been a reflection of concurrent low LA levels in the diet (Ip 1997, Craig-Schmidt et al. 1993). Furthermore, several studies investigating the effects of different dietary fats on carcinogenesis have used diets deficient in the essential n-3 fatty acid LNA (Hussey and Tisdale 1994, Katz and Boylan 1989, Hubbard and Erickson 1987, Kort et al. 1987, Chan et al. 1983). The essentiality of n-3 fatty acids in the body is clear as they play a distinct role in the structure and function of cell membranes (Spector 1999). In addition, most animal model studies to date have focused on individual dietary fats and oils, whereas human dietary fat intake is usually a mixture of lipids from various sources. Thus, many studies have failed to use diets that are representative of normal consumption patterns in humans. It is possible that interactions between different dietary fats and oils could play an important role in the influence of dietary fat on carcinogenesis. More specifically, the interaction between long-chain n-3 PUFAs and the n-6 fatty acid LA may account for discrepancies among previous studies. That is, the availability of LA in the diet may play a critical role in determining the modulation of tumour growth by EPA and DHA. Overall, the optimal dietary n-6/n-3 fatty acid balance that is needed for the inhibitory effect of n-3 fatty acids to be fully expressed requires further investigation.

## 1.3 ANTICARCINOGENIC MECHANISMS OF N-3 FATTY ACIDS

Although the majority of studies investigating the action of fatty acids in carcinogenesis have concluded that they have more impact on tumour promotion than on tumour initiation, the exact mechanism of action has not been clearly established. However, there are several proposed mechanisms through which EPA and DHA might act to elicit their putative anticarcinogenic effects. Most hypotheses postulate that membrane PUFA concentrations affect vital cell membrane functions which may be relevant to the relationship between diet and cancer.

### 1.3.1 Membrane Lipid Composition

Lipids are a vital component of cell membranes. For example, fluidity of the membrane lipid bilayer depends on the phospholipids, amount of cholesterol present, fatty acid chain length and the degree of unsaturation (Innis and Clandinin 1981). The structure of the phospholipid bilayer is shown in **Figure 1.2**.

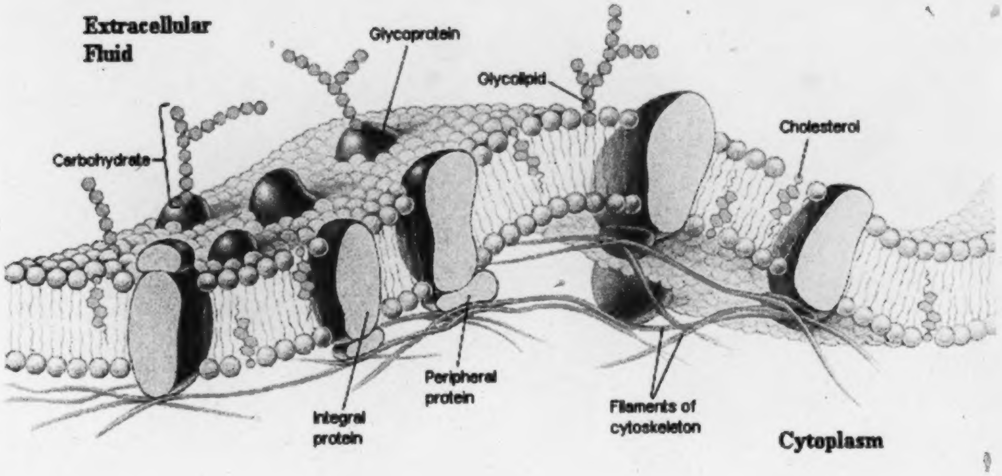

**FIGURE 1.2 Structure of the Phospholipid Bilayer** (reprinted with permission from http://www.people.virginia.edu/~rjh9u/cellmemb.html)

Overall, the fatty acid composition of a cell membrane is a major determinant of its physical properties and also modulates important membrane-dependent cell functions such as integral enzyme activity, intracellular hormone action, membrane receptor function, and eicosanoid production. It is well established that both qualitative and quantitative changes in dietary fat can induce significant alterations in the composition of membranes in a variety of human and animal tissues (Field et al. 1988, Clandinin et al. 1985), including adipocytes (Field et al. 1989), cardiac cells (Innis and Clandinin 1981), hepatocytes (Garg et al. 1988), immune cells (Field et al. 2000, Peterson et al. 1998, Tiwari et al. 1987) and tumour cells (Karmali et al 1984). In turn, such diet-induced changes in membrane fatty acid composition lead to alterations in the function of specific membrane proteins (Clandinin et al 1985). Such changes have been found with adipocyte insulin binding, glucose transport and utilization (Field et al 1989), and ATPase-catalyzed ATP/$^{32}$P $P_i$ exchange (Innis and Clandinin 1981). Cell permeability to various ions is also modified with dietary fatty acids (Devor and Frizzell 1998). In addition, enzymes including Δ6 desaturase (Garg et al 1988) and phosphocholinetransferase (Hargreaves and Clandinin 1987), glucose transporters (Sandra et al. 1984), and second messenger pathways such as protein kinase C (Chapkin et al. 1997), phospholipase C-$\gamma$1 (Jiang et al. 1996), ceramide production and diacylglycerol (Jolly et al. 1997) have been found to exhibit altered activity with changes in dietary fat. Overall, dietary modification of membrane lipids could have important implications for normal cell function and disease, providing a mechanistic link between diet, host immune function, and tumour growth and development.

#### 1.3.1.1 Immune Cells

Dietary lipids have been shown to alter phospholipid profiles of immune cells, such as lymphocytes (Field et al 2000, Peterson et al 1998, Tiwari et al 1987), macrophages (Chapkin and Carmichael 1990), and polymorphonuclear cells (Cleland et al. 1990). Since much of the function of the immune system depends on interactions involving the plasma membrane of both effector and target (eg. tumour) cells, incorporation of dietary lipids into the plasma membrane of such cells could influence immune responses.

#### 1.3.1.2 Tumour Cells

Cell division has been shown to be associated with both membrane fluidity (Savonniere et al. 1996) and increased concentration of unsaturated fatty acids in the cell membrane (Burns and Spector 1994). In particular, LA exists at higher concentrations in membranes of dividing cells (Burns and Spector 1994). In contrast, a low level of LNA in breast cancer tissue is associated with positive axillary lymph node status, the presence of vascular invasion, and provides an indicator of future metastasis (Bougnoux et al. 1994). It is possible that in a disease state such as cancer where rapid cell division is a concern, altering membrane lipid composition might be a means of modifying cell proliferation. As mentioned above, such alterations in membrane lipid composition can be achieved through modulation of dietary fat intake. The fatty acid composition of several tumours has been changed sufficiently to alter some of their properties and functions. Such modifications have been produced *in vivo* by feeding fat-supplemented diets to tumour-bearing animals (Jurkowski and Cave, Jr. 1985) and *in vitro* by adding specific fatty acids to the cell culture media (Burns and Spector 1987).

For example, the influence of dietary fat manipulation on the fatty acid composition of tumour cells has been demonstrated using the NMU-induced mammary tumour model where rats were fed diets containing increasing amounts (0.5%, 3%, or 20% w/w) of fish oil rich in EPA and DHA (Jurkowski and Cave, Jr. 1985). In this study, tumour cells from each of the different diet groups had distinctive lipid compositions related to the dietary fat composition. Specifically, tumour cells from rats fed diets containing fish oil had significantly higher amounts of n-3 fatty acids compared with control corn oil-fed rats, and this effect was greatest in animals fed the 20% (w/w) fish oil diet (Jurkowski and Cave, Jr. 1985).

Overall, cancer cells are sensitive to PUFAs because: (1) essential fatty acid metabolism in cancer cells is abnormal and deficiency in converting parent essential fatty acids exists; (2) cancer cells convert essential fatty acids to different metabolites than those formed in normal tissues. For example, there is impaired conversion of fatty acids to AA and docosapentaenoic acid (C22:5n-3, DPA) in cancer tissue; and (3) there are abnormal fatty acid profiles in the host tissues. It is likely that more than one of these factors are involved in the sensitivity of cancer cells to PUFA (Jiang et al. 1998)

In interpreting results from many earlier studies investigating diet-induced alterations in tumour cell fatty acid composition, the methodologies used must be carefully considered. The ability of dietary long-chain n-3 fatty acids to alter the fatty acyl composition of individual tumour cell membrane phospholipid fractions has not been extensively studied. Such a separation is important because subcellular membrane types contain different contents of phospholipid fractions which respond to dietary fat manipulation to varying degrees (Field et al. 1990). For example, Liu et al. (1994) have demonstrated that the fatty acyl composition of individual phospholipids of skeletal muscle plasma membrane is significantly affected by dietary long-chain n-3 PUFAs and that the content of specific fatty acids responds to dietary manipulation differently in individual phospholipids (Liu et al. 1994). Since dietary n-3 fatty acids have been shown to decrease tumour growth by some unknown mechanism, future research in this area is warranted in an attempt to determine how these fatty acids may be exerting their anticarcinogenic action.

### 1.3.2 The Immune System

The host immune system is a highly complex system which plays a vital role in providing resistance against invading microorganisms and foreign agents. Host defense mechanisms are classified as either innate (natural) or acquired (specific) immune responses (Kuby 1994). The innate immune response provides a first line of defense from foreign agents and involves physical barriers (skin, mucous membranes), chemical means (complement and other humoral factors), phagocytic cells (neutrophils and monocytes/macrophages), and natural killer (NK) cells (Kuby 1994). The acquired immune response may be subdivided into cellular and humoral immunity (**Figure 1.3**). Immune cells found in the bone marrow, thymus, spleen, lymph nodes, and in the blood and lymph circulation include T and B lymphocytes, which are involved in mediating cellular and humoral immune responses, respectively (Kuby 1994).

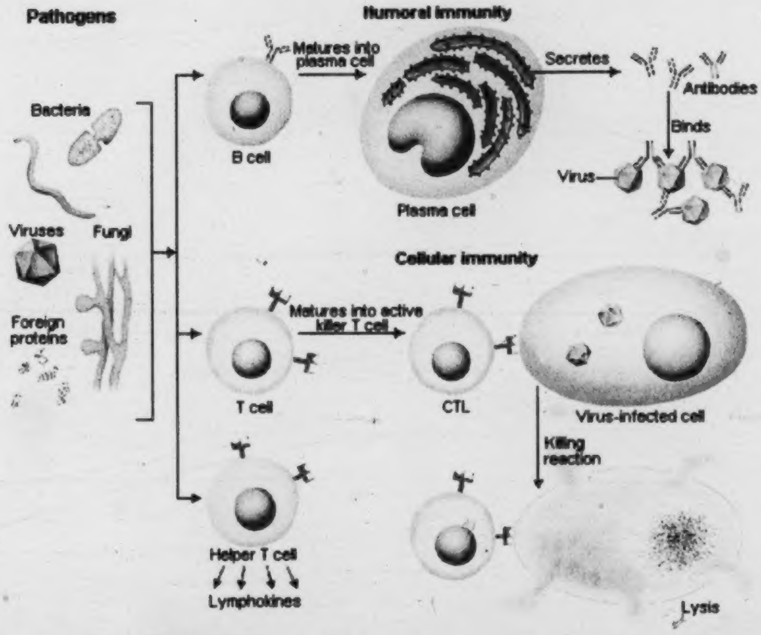
Pathogens
Humoral immunity
Matures into plasma cell
Secretes
Antibodies
Bacteria
B cell
Binds
Virus
Plasma cell
Viruses
Fungi
Cellular immunity
Foreign proteins
Matures into active killer T cell
T cell
CTL
Virus-infected cell
Killing reaction
Helper T cell
Lymphokines
Lysis

**FIGURE 1.3 Humoral and Cellular Immunity** (reprinted with permission from http//www.people.virginia.edu/~rjh9u/cellmemb.html)

### 1.3.2.1 Antitumour Immune Defense

The immune surveillance theory proposes that the immune system is actively involved in detecting and destroying neoplastic cells (Burnet 1970). There are several immune cell populations believed to be involved in this response.

*T cells*

The total T cell population represents the majority of the circulating pool of lymphocytes in blood and lymph and in certain areas of the lymph nodes and spleen, as well as the gut-associated lymphatic system (Kuby 1994). These cells are able to recognize invading antigens by means of specific antigen-binding T cell receptors (TCR) on their membranes. However, such TCR can recognize antigen only in association with cell membrane proteins called major histocompatibility complex (MHC). When a naive T cell encounters antigen associated with an MHC molecule on a cell, the T cell proliferates and differentiates into various effector T cells and a population of memory T cells which act upon reexposure to the same foreign antigen (Kuby 1994).

The two signal model for T cell activation was introduced by Lafferty and Cunningham (1975). The first signal is defined as occupancy of the TCR by a complex formed between antigenic peptide and MHC molecules on the antigen-presenting cell (APC), which are predominantly dendritic cells, but also monocytes, macrophages, and activated B cells. The second costimulatory signal is delivered by soluble costimulatory factors and/or by a ligand molecule on the APC surface that binds to a distinct receptor on the T cell surface. According to this model of T cell activation, T cells triggered through the TCR in the absence of costimulation, become anergic (Schwartz 1990), and do not generate effector functions. Thus, manipulation of costimulatory signals for T cell activation can potentially be used to enhance or induce the immunogenicity of malignant cells (Lanzavecchia 1993).

There are two distinct subpopulations of T cells, T helper (Th) and T suppressor-cytotoxic cells, which can be distinguished by function and by the binding of specific fluorescently-labelled monoclonal antibodies to one of two membrane glycoproteins, either CD4 or CD8. In general, T helper cells express CD4 and recognize antigen associated with Class II MHC molecules, while T suppressor/cytotoxic cells display CD8 and recognize antigen associated with Class I MHC molecules (Kuby 1994).

Much attention in the area of tumour immunology has been given to the role of $CD8^+$ T cells called cytotoxic T lymphocytes (CTLs) because most tumours are MHC class I positive, but negative for MHC class II. Moreover, $CD8^+$ CTLs are able to lyse tumour cells upon recognition of peptide-MHC class I complexes expressed by the tumour, and their ability to eradicate large tumour masses *in vivo* has been demonstrated (Melief 1992). Studies using adoptively transferred purified T cell subsets or *in vivo* depletion studies have verified an important role for tumour-specific $CD8^+$ CTLs in anticancer immune defense (Melief 1992). Recent evidence indicates that $CD4^+$ T cell responses are also a critical component of antitumour immunity (Toes et al. 1999, Pardoll and Topalian 1998). For example, the role of $CD4^+$ Th cells in the priming of CTLs is well documented (Toes et al 1999, Keene and Forman 1982), explaining why activated CTLs, but not naïve CTLs, can mediate potent antitumour effects even in the absence of $CD4^+$ T cells.

### *Cytokines*

Both $CD4^+$ and $CD8^+$ T cells play a central regulatory role in almost all immune responses because of their production of cytokines (Kelso 1998). In particular, T cells can produce different combinations of cytokines, including those classified as type 1, type 2 or shared based on studies with T cell clones (Kelso 1998). Murine memory Th cells have been functionally divided into distinct subsets based on their cytokine secretions (Mosmann and Sad 1996). Briefly, Th1 cells secrete interleukin (IL)-2, interferon (IFN)-$\gamma$ and tumour necrosis factor (TNF)-$\beta$, which are involved in cell-mediated immunity and delayed-type hypersensitivity, while Th2 cells produce other cytokines (IL-4, IL-5, IL-6, IL-10, and IL-13) involved in humoral immune responses and immediate-type hypersensitivity (Mosmann and Sad 1996). Th0 cells secrete a mixed pattern of cytokines (Lucey et al. 1996). Cells in these three subsets differentiate from a common naïve $CD4^+$ Th cell precursor (Mosmann and Sad 1996). In humans, the distinction between these Th subsets is less clear, but clinical conditions with a predominance of either Th1 or Th2 cytokine secretion have been identified (Lucey et al 1996) and T cell clones with cytokine secretion patterns corresponding to murine Th1 and Th2 cells have been generated (Seder et al. 1994).

Th1/Th2 balance is determined early during immune responses and depends on many factors including antigen structure, functional status of APC, strength of T cell activation, presence of cytokines such as IL-12 and IL-4, costimulatory signals such as CD80 or CD86, and the microenvironment (Mosmann and Sad 1996). Theoretically, a Th1 response will result in cell-mediated immunity against tumour cells. However, some tumours produce cytokines, such as IL-10 (Venetsanakos et al. 1997) and prostaglandin E2 (Huang et al. 1996) in their local microenvironment that can direct tumour-infiltrating T cells towards Th2 responses and prevent development of an anticancer Th1 response (Pawelec et al. 1997). In a comparison of spontaneously regressing with nonregressing melanomas, Lowes et al. (1997) found elevated levels of mRNA for Th1 cytokines IL-2, TNF-β, and IFN-γ in the former compared to the latter, although they found no differences in Th2 cytokines IL-10, IL-13, or proinflammatory cytokines IL-1, IL-6, IL-8, TNF-α, or the factors GM-CSF and transforming growth factor-β (Lowes et al. 1997). In some models, successful immunotherapy of established tumours is associated with a change in the balance of T cell subsets from Th2 to Th1 (Gabrilovich et al. 1996).

### *NK Cells*

NK cells, a subpopulation of lymphocytes distinct from T and B cells, represent 5-10% of the mononuclear cells in peripheral blood and spleen and can be identified by the presence of azurophilic granules (Trinchieri 1989). They are an important part of the first line of defense against cancer by recognizing and actively destroying certain tumour cells before T cells and macrophages can be mobilized (Herberman 1984). NK cells do not express the specific antigen-binding receptors of either T or B cells. NK cells demonstrate non-MHC restricted cytotoxicity and exert their effects when MHC Class I molecule expression is reduced (Timonen 1997). However, there appears to be a degree of specificity to NK cell killing, since many virally-infected or tumour cells and most normal cells are not susceptible to NK lysis *in vitro*. The basis of this specificity is not understood. The tumouricidal capacity of NK cells is increased by cytokines, including IFN-γ, TNF-α, IL-2, and IL-12. Therefore, their role in antitumour immunity may depend on the concurrent stimulation of T cells and macrophages that produce these cytokines. NK activity is also regulated by eicosanoid products of AA metabolism, such as prostaglandin $E_2$ and leukotriene $B_4$ (Vaillier et al. 1992). It has been demonstrated that NK cells are able to metabolize AA and to synthesize eicosanoids from both the lipoxygenase and cyclooxygenase pathways (Cifone et al. 1993). Tumour lysis by NK cells is believed to be carried out by perforin, granzymes, and nitric oxide (Filep et al. 1996).

*Macrophages*

Macrophages are a first line of defense against foreign microorganisms and tumour cells by nature of their phagocytic, cytotoxic, and intracellular killing capacities (Herberman 1983). Along with NK cells, they have the ability to destroy tumours without prior exposure to them and may exert a surveillance role against spontaneously arising malignant cells (Herberman 1983). In addition, macrophages are central to the proper function of the immune system by their ability to produce cytokines and by antigen presentation to T cells through class II MHC (Kuby 1994). Although the exact mechanism responsible for macrophage killing of tumour target cells is unclear, it appears that it occurs through both direct and indirect pathways. The indirect pathway involves the release of cytokines (IL-1 and TNF-α) from activated macrophages. Macrophages can also mediate lysis through the release of molecules which are toxic to or inhibit replication of tumour cells, such as nitric oxide (Mills et al. 1992). Support for a direct mechanism of macrophage cytotoxicity arises from the ability of activated macrophages to lyse tumour cells resistant to IL-1 and TNF-α. Macrophages have been shown to exist in higher concentrations around tumours and to destroy neoplastic cells *in vitro* (Herberman 1983).

### 1.3.2.2 Immune Surveillance Theory

Although tumours are derived from self tissues, the process of malignant transformation may be associated with the expression of protein antigens on the tumour cell surface that are recognized as foreign by the host immune system. In fact, tumour antigens may induce immune responses directed at the tumour cells on which they are expressed. Thus, it is possible that the immune system could recognize and destroy abnormal cells before they grow into tumours, or could kill tumours after they are formed. This theoretical role for the immune system is called immune surveillance (Burnet 1970) and involves various effector cells, such as T and B cells, NK cells and macrophages which are able to recognize tumour antigens and mediate the killing of tumour cells (Sheu et al. 1999, Shu et al. 1997).

In general, immune surveillance involves the following 5 steps: *(1) Antigen expression on tumour cells:* Tumour-associated antigens (TAA) are mutant or overexpressed cellular proteins that are bound to membrane surface MHC class I molecules (Parmiani 1993); *(2) Tumour antigen presentation and recognition by T cells:* Macrophages and B cells process TAA that then bind to the T cell receptor (TCR) and a surface MHC class II molecule on $CD4^+$ T helper cells (Boon et al. 1994). This recognition initiates stage I of T cell activation which involves RNA and protein synthesis; *(3) Co-stimulatory signal received for stage II of T cell activation:* Stage II of T cell activation involves DNA and IL-2 synthesis resulting in clonal expansion of T cells (June et al. 1994). The costimulatory signal is derived from the interaction of CD28 on $CD4^+$ T helper cells or CTLA-4 on $CD8^+$ T suppressor/cytotoxic cells with the molecule B7 (CD80) on antigen-presenting cells or tumour cells (June et al 1994). T cell activation in the absence of costimulation induces apoptosis of T cells (June et al 1994). Once activated, T cells no longer require the costimulatory signal for further stimulation; *(4) IL-2 production:* Activated $CD4^+$ T (Th1) cells synthesize and release IL-2, which induces the receptor for transferrin (CD71) (Neckers and Cossman 1983) and stimulates activation and expansion of $CD8^+$ T and NK cells (Ashwell et al. 1994); *(5) Migration of effector cells to tumour:* Immune activation induces adhesion/accessory molecules on B cells (CD80), monocytes (ICAM-1) and T cells (LFA-1, LFA-3, ICAM-1), enabling these effector cells to migrate to the tumour site and initiate tumour cell lysis (June et al 1994).

Unfortunately, immune surveillance is often ineffective, as indicated by the appearance of lethal cancers in immunocompetent individuals. Therefore, it is likely that many of the immune responses against tumours are weak or blocked by the tumour (Kiessling et al. 1999). Thus, a major focus of current research in immunology and oncology is the development of methods to augment host antitumour immune defense.

#### 1.3.2.3 Tumour Evasion of the Immune System

Although systemic adjuvant therapy significantly improves survival for many patients (Early Breast Cancer Trialists' Collaborative Group 1992) approximately 50% of women with apparently localized breast cancer will ultimately die of metastatic disease (Ellis and Smith 1996). One possible explanation for this poor prognosis is the failure of standard treatment to destroy all cancer cells and the ability of remaining malignant cells to escape detection and destruction by the host immune system. Various mechanisms have been proposed to explain how antigen expressing tumor cells evade the immune system (Pawelec et al 1997). These mechanisms include: *(1) loss or down-regulation of class I MHC molecules on tumor cells* (Garrido et al. 1993); *(2) down-regulation or lack of costimulatory signal* (Chen et al. 1993); *(3) tumor-derived immunosuppression through the synthesis of molecules such as IL-10 and transforming growth factor-β* (Venetsanakos et al 1997, Maeda and Shiraishi 1996).

### 1.3.3 Dietary Fat and Immune Function

Both *in vivo* and *in vitro* evidence suggests that dietary fat plays a role in modulating immune function (Kinsella and Lokesh 1990). For example, essential fatty acid deficiency has been associated with suppressed cell-mediated immune responses, reduced phagocyte activity, and decreased antibody production (Johnston and Marshall 1984) Similarly, excessive dietary fat intake has been shown to suppress T cell proliferation and thus inhibit cell-mediated immunity (Calder 1998). Many studies have also demonstrated that the type of fat in the diet can have a major impact on cell-mediated immunity. In particular n-6 and n-3 PUFAs have generated considerable interest for their potential to modulate immune function (Calder 1998, Yaqoob 1998). Numerous animal studies have demonstrated that fish oil-derived long-chain n-3 fatty acids modulate a wide range of immune responses, including lymphocyte proliferation, delayed-type hypersensitivity reactions, and antigen presentation (Calder 1998, Yaqoob 1998). Clinical studies have also shown that consumption of fish oils can have beneficial effects on inflammatory and autoimmune disorders, such as rheumatoid arthritis, asthma, psoriasis, and cystic fibrosis (Harbige 1998). While it is widely recognized that dietary fats can alter immune and inflammatory responses, current understanding of how dietary lipids change the immune response is incomplete. One principal mechanism by which dietary fat may influence immune responsiveness is through diet-induced alteration of immune cell membrane structure and function as discussed above.

It has been demonstrated that feeding fish oil to rabbits (Kelley et al. 1988), chickens (Fritsche et al. 1991), rats (Sanderson et al. 1995, Yaqoob et al. 1994), and mice (Yaqoob and Calder 1995) results in suppressed T cell proliferation compared with feeding hydrogenated coconut, safflower, corn or linseed oils or lard. However, the extent of inhibition reported by different studies using the same type of oil is variable. Such discrepancies are most likely due to a variety of effects, including differences in amount of dietary fat, duration of feeding, species and strain of animal used, source of the lymphocytes studied, mitogen and its concentration, and type of serum used for culturing lymphocytes *ex vivo*.

In contrast to the large number of studies investigating the effects of dietary fish oil on the *ex vivo* production of macrophage-derived cytokines, there have been relatively few studies on lymphocyte-derived cytokines. One study reported increased IL-2 and IL-4 production by Con A-stimulated splenocytes isolated from autoimmune disease-prone mice fed 100 g/kg fish oil compared with those fed 100 g/kg corn oil (Fernandes et al. 1994). In another study, the concentrations of IL-2, IL-4, IL-10, and IFN-γ in the culture medium of Con A-stimulated murine spleen lymphocytes were not significantly affected by diet fat (Yaqoob and Calder 1995).

Overall, it has been well documented that feeding high levels of n-3 fatty acids significantly affects the functions of immune cells tested *ex vivo*. However, there are many contradictory observations regarding the influence of both n-6 and n-3 fatty acids on immune parameters. It is likely that these discrepancies result from the wide variety of experimental protocols used. Although some of the effects of n-3 fatty acids may be associated with modulation of the amount and types of eicosanoids produced, it is possible that eicosanoid-independent mechanisms, including actions upon intracellular signalling pathways and transcription factor activity, are also involved (Hwang and Rhee 1999)

Although many studies have shown that dietary fat affects the immune system, relatively limited data are available on how this, in turn, influences experimental carcinogenesis. However, certain studies have shown that diets rich in n-6 fatty acids (primarily LA) increase tumour growth through processes mediated by depression of cell-mediated immune responses (Thomas and Erickson 1985). The issue of the role of dietary fat in the modulation of immune function during cancer is not without debate. For example, a study by Welsch et al. (1993) showed that a high fat diet consisting of 19% (w/w) fish oil + 1% (w/w) corn oil significantly inhibited growth of human breast carcinomas (MDA-MB-231 and MCF-7) maintained in immune deficient mice (beige-XID-athymic nude mice and severe combined immune-deficient mice). Based on these results, it was concluded that tumour growth suppression induced by dietary fish oil was not mediated by immune system mechanisms involving T, B, NK cells or macrophages. Thus, although diet-induced immune system modulation is one possible mechanism responsible for the effects of dietary fatty acids on tumour growth, other mechanisms must also be involved. That is, dietary lipids may affect tumour development and growth directly, through changes in tumour cells. This is supported by evidence that various types of fatty acids affect growth of tumour cells when added to culture media *in vitro* (Awad et al. 1996, Rose and Connolly 1990). Clearly, these effects are not associated with immunological mechanisms.

### 1.3.4 Fatty Acids and Tumour Cell Growth *In Vitro*

LA has been reported to have either no effect on growth of tumour cells *in vitro* (Grammatikos et al. 1994), to stimulate (Rose and Connolly 1990), or to inhibit tumour growth (Gardiner and Duncan 1991). These diverse effects of LA may depend on the tumour type. For example, in most of the studies, breast cancer cell proliferation was stimulated by LA, whereas growth of melanoma, colorectal and liver cancer cells was suppressed by LA and that of pancreatic and prostate cancer cells was either stimulated or inhibited (Jiang et al 1998).

The ability of n-3 fatty acids to inhibit cell proliferation *in vitro* has been well documented (Awad et al 1996, Grammatikos et al 1994, Begin et al. 1986). For example, Grammatikos et al. (1994) studied the growth response of MCF-7 human breast cancer cells to the n-6 fatty acids, LA and AA and the n-3 fatty acids, LNA, EPA, and DHA. While both EPA and DHA inhibited growth of MCF-7 cells by as much as 50% in a dose dependent manner, LNA and AA inhibited growth less extensively, and LA had no effect (Grammatikos et al 1994). It is well established that PUFA, including EPA and DHA, can be toxic to tumour cells, while being much less toxic or non-toxic to normal cells (Jiang et al 1998). In the study by Grammatikos et al. (1994), the observed growth inhibitions were not due to fatty acid-induced cytotoxic effects, since cell viability did not drop below 99% and a 2- to 3-fold expansion in cell number was obtained even at the highest levels of fatty acids studied. Using the MDA-MB-231 human breast cancer cell line, Rose and Connolly (1990) showed that DHA was more effective than EPA in suppressing tumour cell growth. Specifically, DHA supplementation (2 μg/mL) reduced cell number by 50%. Trypan blue exclusion indicated that the cells present were viable, suggesting that cell loss was due to inhibition of mitosis rather than cytotoxic cell death. Chajes et al. (1995) also found that growth arrest of MDA-MB-231 breast cancer cells by EPA and DHA was not due to major cytotoxic effects. Thus, although nonspecific cytotoxicity does not appear to be responsible for growth inhibition induced by n-3 fatty acids at low concentration, at high levels, both n-3 and n-6 fatty acids caused lysis of human breast cancer cells *in vitro* (Begin et al. 1988, Begin and Ells 1987, Begin et al 1986). However, both the physiological and clinical relevance of *in vitro* cell culture experiments performed with very high fatty acid concentrations must be questioned. Furthermore, while the inhibition of cell proliferation by n-3 fatty acids *in vitro* has been well documented, the cellular and molecular changes induced by n-3 fatty acids remain poorly understood.

#### 1.3.4.1 Fatty Acids and Apoptosis

Increasing cell number and formation of a solid tumour is the result of excessive cell proliferation, insufficient programmed cell death (apoptosis), or a combination of these two cellular events (Weinberg 1996a). As described previously, apoptosis is a carefully regulated biochemical process in which cells are programmed to die under a range of physiological and developmental factors (Saikumar et al 1999). Although there is direct evidence that the long-chain n-3 fatty acids can induce apoptosis, this appears to be cell-type specific. Thus, while EPA resulted in apoptosis in a human pancreatic cancer cell line (Lai et al. 1996), this n-3 fatty acid inhibited Morris hepatocarcinoma 3924A growth *in vivo* primarily due to reduced proliferation. In contrast, DHA induced apoptosis in this model (Calviello et al. 1998).

A study by Gabor & Abraham, which predated the current intense interest in apoptosis and cancer, found that the inhibition of growth of a transplantable mouse mammary adenocarcinoma by dietary n-3 fatty acids was a result of cell loss, rather than suppressed proliferation. This was the first study to infer the effects of n-3 fatty acids on breast cancer cell apoptosis. Recently, Connolly et al. (1999) showed that DHA-induced inhibition of MDA-MB-231 breast cancer cell growth *in vivo* was associated with both a partial suppression of cell proliferation and increased apoptosis. Thus, this study concluded that DHA-induced growth inhibition is likely multifactorial, including both suppression of cell proliferation and enhancement of apoptotic cell death.

#### 1.3.4.2 Summary

*In vitro* studies of tumour cells cultured in media supplemented with various types and amounts of fatty acids can provide information regarding tumour cell growth and corresponding molecular changes. The influence of fatty acids on tumour growth in these assays is directly on tumour cells rather than on the whole tumour or on some aspect of the whole animal host, such as the immune system. However, since *in vitro* fatty acid studies do not assess the influence of the entire diet on tumour promotion, this supplementation method is not representative of fatty acids found in human diets. In an attempt to fully establish the influence of dietary fat on tumour growth, it is necessary to use animal models representative of human cancer in addition to tumour cell lines.

## 1.4 EXERCISE AND IMMUNITY

### 1.4.1 Overview

Increasing evidence from both epidemiological and laboratory-based investigations suggests that exercise may affect cancer incidence and progression (Woods 1998, Bernstein et al. 1994). For example, certain animal studies have shown that exercise can slow the progression of chemically-induced, transplantable, and/or spontaneous tumours (Welsch et al. 1995). In contrast, other studies have shown little, if any beneficial, effects of exercise on transplantable tumours and intense exercise can even have negative effects on tumour growth (Woods et al. 1994). Overall, however, the currently available evidence from animal studies supports a role for exercise of moderate-intensity in host resistance to tumourigenesis (Woods 1998). For example, a low-intensity swimming program in rats implanted with the Morris Hepatoma 7777 decreased tumour growth (Baracos 1989). One possible mechanism by which exercise may affect tumour growth is through its potential to alter antitumour immune responses (Woods et al 1994).

Both human and animal studies investigating the effects of exercise on the immune system have found that exericise-induced immunomodulation occurs due to altered composition of immune cells as well as due to changes in immune function. More specifically, parameters such as the absolute number of circulating immune cells, the relative proportion of lymphocyte populations, and the function of macrophages, NK, T, and B cells may be altered with exercise (Ndon et al. 1992). However, controversy exists over whether exercise affects these immune parameters positively or negatively, thereby leading to stimulation or suppression of the immune system. It has been suggested that both the intensity and duration of exercise are key factors in determining how exercise influences the immune system (Woods et al 1994). For example, evidence suggests that high-intensity exercise leads to immune suppression, while exercise of moderate intensity may enhance the immune system (Brines et al. 1996).

### 1.4.2 Acute Exercise

To date, many researchers studying the immune response to exercise have focused on acute immunological changes following a single bout of exhaustive exercise. From these studies, it has been well established that acute intensive exercise induces transient immunosuppression as indicated *in vitro* by reduced mitogen-stimulated proliferation of lymphocytes and also decreased CD4/CD8 T cell ratios (Woods et al. 1999). Such responses to acute exercise have been found to occur despite an increase in the total number of immune cells in peripheral circulation (Field et al. 1991). Alterations in the immune response to acute exercise are usually transient; most of the observed changes return to pre-exercise levels within 2-24 h post-exercise (Ndon et al 1992, Field et al 1991). Although the clinical significance of this exercise-induced immunomodulation is not yet known, it has been suggested that actue exhaustive exercise may lead to adverse alterations in immune function and increased susceptibility to disease (Hoffman-Goetz and Pedersen 1994). However, considering that most immunological responses to acute exercise are short in duration, it is likely that this type of exercise will have only a minor impact on host immune defense mechanisms against diseases, such as cancer. More significant effects of exercise on antitumour immune defenses may be those changes in immune response which occur during or following chronic exercise-training.

### 1.4.3 Chronic Exercise-training

The persistant changes in the structure and function of the immune system following chronic exercise-training (involving bouts of intense exercise) have been widely studied and have been associated with both deleterious and beneficial effects on immunocompetence (Hoffman-Goetz and Pedersen 1994, Lin et al. 1993). Overall, studies on the immunological consequences of high-intensity chronic exercise-training have suggested that such vigorous training is related to adverse alterations in immune function (Woods et al 1999, Nieman 1997). For example, studies on elite endurance athletes have suggested that prolonged intense exercise can have long-term damaging effects on the immune system (Fitzgerald 1988).

There are relatively fewer studies on the immunomodulatory effects of low to moderate intensity chronic exercise-training, such as that experienced by an individual performing regular non-exhaustive exercise. Although the general consensus is that regular exercise of low to moderate intensity may improve the ability of the immune system to protect the host against infection (Hoffman-Goetz and Pedersen 1994), few studies have used appropriate exercise protocols to examine this hypothesis. Many previous animal studies investigating the effects of exercise on immune function have used running protocols of long duration and high intensity (Hoffman et al. 1962, Rusch and Kline 1944). The results of these studies may have been confounded by the high stress levels induced by using such severe exercise regimes (Steplewski et al. 1985).

Shewchuk et al. (1997) found that chronic low-intensity swimming in rats did not alter the number or proportion of immune cell types present in spleen (Shewchuk et al. 1997). This finding agrees with that reported for human peripheral blood immune cells after moderate-intensity exercise-training (Nieman et al. 1993). Furthermore, while the mitogenic response of lymphocytes after acute exercise is quite consistent, showing a decreased proliferation of immune cells in both animals and humans, the proliferative response of lymphocytes following chronic exercise-training shows little consistency. That is, increases, decreases, and no change in mitogenic respose following exercise-training have been reported (Tharp and Preuss 1991). Several studies on animals and humans have shown significant improvements in NK cell cytotoxic activity with exercise-training (Hoffman-Goetz et al. 1992). However, the mechanism underlying this higher NK activity has not yet been established.

## 1.5 DIETARY AMINO ACIDS

Since anticancer immune defense declines progressively with tumour growth (Pawelec et al 1997), an important clinical goal is identifying means of stimulating immune function during cancer. Nutritional support is one such means of reversing immunosuppression in the tumour-bearing host. Specific nutrients such as glutamine, arginine and ornithine $\alpha$-ketoglutarate (OKG) have attracted recent attention for their immunoenhancing properties (De Bandt and Cynober 1998). The potential benefit of supplementation with specific nutrients has aroused great interest due to their effects on immune function. The results of many of these studies have been used to formulate multiple nutrient 'immune boosting' commercial formulas (Atkinson et al. 1998, Kemen et al. 1995). These formula containing many "immunonutrients" have deterred the ability to draw conclusions as to the efficacy of each individual nutrient.

### 1.5.1 Glutamine

The nonessential amino acid glutamine is a major source of energy and nitrogen for protein and nucleotide synthesis in rapidly dividing cells such as intestinal mucosal cells (Souba et al. 1985, Windmueller and Spaeth 1980) and immune cells (Shewchuk et al. 1996, Newsholme et al. 1985). During catabolic disease states, an increased glutamine utilization co-exists with intestinal atrophy (Jacobs et al. 1988), immunosuppression (Alverdy 1990, Newsholme et al 1985), and reduced protein synthesis (MacLennan et al. 1987). Thus, in situations of surgery, trauma or sepsis, glutamine supplementation has been shown to improve immune function, nitrogen balance, and nutritional status (Wernerman and Hammarqvist 1999, Yoshida et al. 1998, Newsholme et al 1985). This suggests that glutamine is an essential nutrient during catabolic states which could be of potential benefit to cancer patients. A randomized, double-blind study by Zeigler et al. (1992) was the first to examine the effect of glutamine-supplementated total parenteral nutrition (TPN) on clinical outcome. This study reported improved nitrogen balance, fewer clinical infections, and shorter hospital stay in patients receiving supplementary glutamine via TPN after bone marrow transplantation (Ziegler et al. 1992).

Glutamine depletion occurs in blood and tissue free pools during the growth of a variety of tumours (Shewchuk et al 1996, Kaibara et al. 1994). In tumour-bearing animals, muscle tissue is repleted when TPN is supplemented with glutamine (Klimberg et al. 1990). In addition, chemotherapy-induced insults to the gastrointestinal tract, such as enterocolitis, endotoxin translocation (Fox et al. 1988), and mucosal damage are minimized with glutamine supplementation (Decker-Baumann et al. 1999, O'Dwyer et al. 1989, Jacobs et al 1988). However, in spite of these beneficial effects of glutamine supplementation, some evidence has suggested that glutamine may provide an energy source for rapidly dividing tumour cells cells (Ardawi 1988, Ardawi and Newsholme 1985). In contrast, *in vivo* studies have demonstrated decreased tumour growth with oral glutamine supplementation (Shewchuk et al 1997, Rouse et al. 1995, Fox et al 1988). The results of these studies suggest decreased tumour growth was due to provision of glutamine as a substrate for energy metabolism and protein synthesis by immune cells (Shewchuk et al 1997, Klimberg and McClellan 1996). Overall, evidence suggests host protection when glutamine is included in dietary formulations for the critically ill due to its roles in gastrointestinal integrity and immune function.

### 1.5.2 Arginine

Arginine is another nonessential amino acid which may have beneficial effects during catabolic states. Arginine is utilized by all tissues for protein synthesis and, like glutamine, there is an increased use of this amino acid during septic states (Kirk and Barbul 1990). Arginine supplemented enteral feeds were shown to decrease the incidence of infections and wound complications after major surgery in humans (Daly et al. 1988) perhaps due to arginine-induced stimulation of lymphocyte mitogenesis (Barbul 1990, Reynolds et al. 1988). In addition, animal studies have shown that arginine increases macrophage and NK cell cytotoxicity as well as IL-2 production (Barbul 1990), which are important anticancer immune responses. In tumour-bearing animals, reduced tumour growth and enhanced host survival have been reported following dietary arginine supplementation (Reynolds et al. 1990). A possible mechanism of action of arginine is its role as a precursor for the multifunctional mediator, nitric oxide (Salvemini et al. 1989). Nitric oxide, and equal amounts of citrulline, are synthesized from L-arginine by nitric oxide synthase (Cifone et al. 1995). Secreted at high levels by activated macrophages and neutrophils, nitric oxide is an important cytotoxic effector molecule in the defense against tumour cells (Kolb and Kolb-Bachofen 1992). However, supplemental arginine can augment tumour growth in some animal models and human tumours (Edwards et al. 1997, Grossie, Jr. 1996, Park et al. 1992, Yeatman et al. 1991).

### 1.5.3 Ornithine α-ketoglutarate (OKG)

OKG, an ionic salt, has a role in protein metabolism because of its two components, ornithine and α-ketoglutarate which belong to the urea cycle and Krebs cycle, respectively (Cynober 1995). OKG has been proposed for the treatment of hypercatabolic states, including trauma, burns, and chronic malnutrition (Donati et al.

1999). When used as an adjuvant in enteral nutrition, OKG improves nitrogen balance in catabolic states (Cynober 1991). For example, in surgical patients, OKG improved nitrogen balance, reduced urinary 3-methylhistidine excretion, increased muscle protein synthesis, and prevented a postoperative decrease in muscle glutamine pools (Hammarqvist et al. 1990). OKG may be a suitable precursor for the synthesis of several metabolites that become depleted in stress situations, including the nonessential amino acids glutamine, arginine, and proline and polyamines (putrescine, spermidine and spermine) and/or stimulation of secretion of anabolic hormones, such as insulin or growth hormone. (Cynober 1991). These OKG metabolites play a role in maintaining protein homeostasis as well as in increasing lymphocyte counts, mitogenic response, synthesis of immunoglobulins, morphonuclear cell function and macrophage antitumour function. However, the use of OKG in cancer is subject to special consideration related to the possible effects of OKG on the tumour. Ornithine, α-ketoglutarate, and their metabolites may be accessible to tumour cells as substrates for energy metabolism or biosynthesis and proliferation (Heys et al. 1991). However, Le Bricon et al. (1994) have demonstrated that enterally fed OKG does not influence *in vivo* growth of Yoshida ascites hepatoma.

There is limited literature on the immune effects of dietary OKG. However, OKG supplementation in surgical patients, elderly, burn patients, endotoxemic and infected rats has been demonstrated to result in fewer infections (Roch-Arveiller et al. 1996). Le Bricon et al. (1995) demonstrated that supplementation with OKG improved nitrogen balance and accelerated amino acid deposition in muscle and gut after surgical tumour removal. Although OKG has not been as widely studied as glutamine and arginine for its immunomodulatory properties, it clearly has potential as an immunoenhancing agent as it may be metabolized to form both glutamine, arginine, and polyamines.

## 1.6 CONCLUSIONS

There is increasing evidence implicating both dietary fat and exercise as crucial factors in modulating tumour growth *in vivo*. In particular, animal research suggests that dietary long-chain n-3 fatty acids inhibit tumour growth. Similarly, evidence from animal models supports a role of low- to moderate-intensity exercise-training in host resistance to cancer. The cellular and molecular basis of these effects are not known. It is possible that the effects of dietary fat and/or exercise are mediated indirectly, through alterations in the host immune system. However, the complex relationships among diet, exercise, and immunity are not currently understood. Since it is well known that anticancer immune defense declines progressively with tumour growth, another important clinical goal is identifying means of stimulating immune function during cancer. Specific nutrients such as n-3 fatty acids, and the amino acids, glutamine, arginine, and OKG, have attracted recent attention for their potential immunoenhancing properties.

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# 2. STUDY RATIONALE

## 2.1 INTRODUCTION

The effect of dietary fatty acids on tumour development and growth has been studied in a variety of carcinogen-induced, transplantable, spontaneous, and metastatic animal tumour models (Ip 1997). Although it is widely accepted that high dietary fat consumption is associated with several types of cancer in animal models, there is increasing evidence that certain types of dietary fat may be anticarcinogenic. For example, many studies have shown that diets rich in the n-6 fatty acid, linoleic acid (C18:2n-6, LA), stimulate tumour growth, whereas diets containing high levels of fish oil-derived long-chain n-3 fatty acids, eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), inhibit it (Ip 1997). Current evidence indicates that the interaction between dietary long-chain n-3 fatty acids and LA is an important factor in determining the modulation of tumour growth by n-3 fatty acids (Ip 1997). The optimal dietary n-6/n-3 fatty acid balance needed for the inhibitory effect of n-3 fatty acids to be fully expressed has not been established. Furthermore, previous research has not often considered the effects of dietary fat level and composition on the efficacy of long-chain n-3 fatty acids nor attempted to formulate diets representative of normal consumption patterns in humans. These considerations are imperative if the anticancer effects of EPA and DHA observed in animal models are to be extrapolated to recommendations for the human population.

Although the majority of studies investigating the action of dietary fatty acids in carcinogenesis have concluded that they have more impact on tumour promotion than on tumour initiation, the exact mechanism of action has not been clearly established. However, there are several proposed mechanisms through which EPA and DHA might act to elicit their putative anticarcinogenic effects. Most hypotheses postulate that membrane polyunsaturated fatty acid concentrations affect vital cell membrane functions which may be relevant to the relationship between diet and cancer. Thus, in turn, many of the proposed mechanisms are associated with the ability of diet fat to alter the composition and function of cellular membranes. This effect has been well established in a variety of human and animal tissues (Field et al. 1988, Clandinin et al. 1985), including immune cells

(Field et al. 2000, Peterson et al. 1998, Hosack-Fowler et al. 1993) and tumour cells both *in vitro* (Awad et al. 1996, Grammatikos et al. 1994) and *in vivo* (Rose et al. 1995, Jenski et al. 1993, Jurkowski and Cave 1985, Karmali et al. 1984). It is possible that dietary fatty acids affect tumour growth either indirectly, through alterations in the host immune system, or directly, through changes in tumour cell metabolism. Overall, dietary modification of cellular membrane lipids could have important implications for normal cell function and disease, providing a mechanistic link between diet, host immune function, and tumour growth.

Components of both the innate (natural) and acquired immune systems are important contributors to host defense aginst invading pathogens and spontaneously arising tumour cells (Whiteside and Herberman 1995, Robins 1986). Immune cell activation in both of these systems is affected by diets rich in long-chain n-3 fatty acids, such as EPA and DHA (Calder 1999, Yaqoob 1998). However, the physiological relevance of these effects is not clear, since many of these studies involved diets that were deficient in the essential n-6 fatty acid, LA, and that contained levels of n-3 fatty acids far above those consumed by most humans. The specific dietary balance of long-chain n-3 fatty acids (EPA and DHA) and n-6 fatty acids (LA) necessary to promote anticancer immune defense has not been established. Exercise has also been shown to modulate anticancer immunity (Woods 1999). However, many studies in the exercise immunology area have not controlled for dietary fat, a factor which is known to modulate immune function. Thus, the complex relationships among diet, exercise, and immunity are not understood.

Although diet-induced immune system modulation is one possible mechanism for the effect of dietary fatty acids on tumour growth, other mechanisms must also be involved. For example, various types of fatty acids have been shown to affect growth of tumour cells *in vitro* when added to the culture media (Awad et al. 1996, Grammatikos et al. 1994). These effects appear not to be associated with immunological mechanisms and suggest that dietary lipids affect tumour growth through direct changes in the tumour cells themselves. Although the inhibition of tumour cell proliferation by n-3 fatty acids *in vitro* has been well documented, the molecular changes induced by these fatty acids remain poorly understood. It is possible that diet-induced changes in tumour cell lipid composition may alter membrane-dependent functions involved in cellular growth-related processes, such as proliferation and apoptosis.

Anticancer immune defense declines progressively with tumour growth. Thus, a major focus of current research in immunology and oncology, and an important clinical goal, is the development of methods to enhance host antitumour immune defense. Recent investigation has focused on supplementing enteral or total parenteral nutrition with various immunoenhancing nutrients, including amino acids (Shewchuk et al. 1996, Roch-Arveiller et al. 1996), either alone or as part of multiple nutrient formulations (Braga et al. 1999, Atkinson et al. 1998, Kemen et al. 1995). In particular, dietary supplementation with glutamine (Gln), arginine (Arg), or ornithine $\alpha$-ketoglutarate (OKG, a glutamine and arginine precursor) has attracted recent attention for potential to improve anticancer immune function, but these compounds have not been compared systematically in an internally controlled study. Thus, their relative efficacy for upregulating host immune function during cancer is difficult to estimate.

## 2.2 OBJECTIVES AND HYPOTHESES

The overall aim of this research is to provide a mechanism-based rationale for recommendations regarding dietary fat composition, amino acid supplementation and exercise-training during cancer. The objectives of this research are to test the following general working hypotheses:

I. Dietary fat composition alters the fatty acid composition of pre-activated and activated immune cells.

II. The tumour-bearing state alters the fatty acid composition of pre-activated and activated immune cells and affects host immune function.

III. Dietary long-chain n-3 fatty acids inhibit tumour growth through an upregulation of membrane-dependent immune defenses, including immune cell activation.

IV. Dietary long-chain n-3 fatty acids inhibit tumour growth through a reduction in cellular proliferation, an increase in apoptosis, and an alteration in expression of growth-related proteins.

V. Dietary Gln, Arg, and OKG upregulate anticancer immune function in the tumour-bearing state.

## 2.3 CHAPTER FORMAT

The hypotheses posed were tested in a sequence of experiments. These experiments are organized as thesis chapters and have been prepared for submission for scientific publication as individual papers.

### 2.3.1 Study One

It is hypothesized that:

1. Dietary long-chain n-3 fatty acids will increase n-3 fatty acid incorporation into immune cell membrane phospholipids.

2. Exercise-training will alter the fatty acid composition of immune cell membrane phospholipids.

3. Dietary long-chain n-3 fatty acids fed at a physiological level which could potentially be achieved in the human diet will enhance anticancer immune defense, including cell activation and natural killer (NK) cell cytotoxicity, and production of cytokines and nitric oxide.

4. Exercise-training of low-intensity will enhance anticancer immune defense, including immune cell activation and NK cell cytotoxicity.

5. The combination of dietary long-chain n-3 fatty acids and low-intensity exercise-training will further enhance anticancer immune defense (i.e. they will have a synergistic benefit on immune defense).

6. The fatty acid composition of immune cell membrane phospholipids will be altered with mitogen activation. The diet-induced increase in n-3 fatty acid incorporation in immune cell phospholipids will be maintained in an activated state.

**Chapters 3** examines the individual and combined effects of dietary fish oil-derived long-chain n-3 fatty acids and low-intensity exercise-training on fatty acid incorporation into immune cell membranes *(hypothesis 1-2)* and anticancer immune defense in healthy rats *(hypothesis 3-5)*.

**Chapter 4** examines the effects of dietary fish oil-derived long-chain n-3 fatty acids on fatty acid incorporation into immune cell membranes in the pre-activated and activated state *(hypothesis 6)* and further investigates the effects of long-chain n-3 fatty acids on membrane-mediated immune defense in healthy rats *(hypothesis 3)*.

### 2.3.2 Study Two and Three

It is hypothesized that:

7. Dietary long-chain n-3 fatty acids will inhibit mammary tumour growth when fed at a physiological level which could potentially be achieved in the human diet.

8. The efficacy of dietary long-chain n-3 fatty acids to reduce tumour growth will differ with the polyunsaturated/saturated fatty acid (P/S) ratio of the diet. Specifically, long-chain n-3 fatty acids will be more effective at inhibiting tumour growth when fed in a low P/S, compared with a high P/S, diet.

9. Dietary long-chain n-3 fatty acids exert their cancer inhibitory effect through upregulation of the host immune system, including enhanced cell activation, natural killer cell cytotoxicity, and production of cytokines and nitric oxide.

10. The efficacy of dietary long-chain n-3 fatty acids to improve immunity will differ with the P/S ratio of the diet. Specifically, the upregulation of anticancer immune defense by dietary long-chain n-3 fatty acids will be enhanced when fed in a low P/S, compared with a high P/S, diet.

11. The incorporation of n-3 fatty acids into mammary tumour cell membrane phospholipids will be enhanced when long-chain n-3 fatty acids are fed in a low P/S, compared with a high P/S, diet.

12. Dietary long-chain n-3 fatty acids will increase n-3 fatty acid incorporation into immune cell membranes in the pre-activated and activated state in both healthy and tumour-bearing rats.

13. The fatty acid composition of immune cell membrane phospholipids in the pre-activated and activated state will be altered in rats implanted with a mammary tumour compared with healthy rats.

14. Host immune function will be suppressed in rats implanted with a mammary tumour compared with healthy rats.

**Chapter 5** details the findings from Study Two and Three. In Study Two, experiments were designed using a rat mammary tumour model to determine if fish oil-derived long-chain n-3 fatty acids upregulate antitumour immune defense *(hypothesis 9)* and inhibit tumour growth *(hypothesis 7)* when fed as part of a high polyunsaturated fat diet, representative of that currently recommended by several health agencies for the human population. The objective of Study Three was to determine if the addition of long-chain n-3 fatty acids to a low polyunsaturated fat diet, representative of that currently consumed by a large segment of the North American population, would enhance anticancer immune defense and suppress tumour growth in a rat mammary tumour model *(hypothesis 7-10)*. The effect of long-chain n-3 fatty acids fed in either a high or low polyunsaturated fat diet on individual phospholipid fractions in tumour cell membranes was also studied *(hypothesis 11)*.

**Chapter 6** examines the influence of tumour burden and dietary long-chain n-3 fatty acids on the fatty acid composition of host immune cell membranes in the pre-activated and activated state *(hypothesis 12-13)*. The influence of both the tumour and dietary long-chain n-3 fatty acids on membrane-mediated host immune defense is also investigated *(hypothesis 9 and 14)*.

### 2.3.3 Study Four

It is hypothesized that:

15. The addition of fatty acids to the tumour cell culture media will modify the fatty acid composition of tumour cell membrane phospholipids.

16. Incubation of breast cancer cells with the long-chain n-3 fatty acid, DHA, will reduce cellular proliferation relative to control, but will not be cytotoxic to the cancer cells.

17. The inhibitory effect of DHA on tumour cell proliferation is not due to a deficiency of LA in the cell culture media. Specifically, the addition of DHA plus LA to the cell culture media will reduce tumour cell proliferation relative to control.

18. The inhibition of tumour cell proliferation by DHA will be related to cell cycle arrest and an increase of tumour cells in $G_0$ (quiescent phase).

19. The addition of DHA to the cell culture media will decrease the expression of various cell cycle regulatory proteins, such as the D-type cyclins and cyclin B1.

20. The inhibition of tumour cell proliferation by DHA will be related to decreased expression of proliferation-related proteins, such as proliferating cell nuclear antigen (PCNA) and proliferation-related kinase (PRK).

21. The inhibition of tumour cell proliferation by DHA will be related to increased expression of tumour-suppressor proteins, such as p53 and retinoblastoma protein (pRb).

22. Incubation of breast cancer cells with DHA will increase apoptosis relative to control.

**Chapter 7** describes experiments designed in a human breast cancer cell culture model to determine the effects of fatty acids on tumour cell proliferation *(hypothesis 16-17)*, apoptosis *(hypothesis 22)* and the fatty acid composition of cell membrane phospholipids *(hypothesis 15)*. In addition, the effects of fatty acids on the distribution of tumour cells in the cell cycle phases *(hypothesis 18)*, expression of cyclin proteins *(hypothesis 19)* and cell cycle/proliferation- and apoptosis-related proteins *(hypothesis 20-21)* were studied.

### 2.3.4 Study Five

It is hypothesized that:

23. Host immune function will be suppressed in tumour-bearing rats compared with healthy rats.

24. Dietary supplementation with Gln, Arg, and OKG will reduce tumour growth compared with a diet containing non-essential amino acids.

25. Dietary supplementation with Gln, Arg, and OKG will upregulate anticancer immune defenses, such as immune cell activation, the cytotoxic activity of marcophages and natural killer cells, and the production of cytokines and nitric oxide.

**Chapter 8** examines experiments designed in a rat tumour model to compare the relative efficacy of dietary Gln, Arg, and OKG to improve host anticancer immunity *(hypothesis 23 and 25)* and reduce tumour growth *(hypothesis 24)*.

**Chapter 9** summarizes the findings of these hypotheses and provides general discussion.

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# 3. DIETARY LONG-CHAIN n-3 FATTY ACIDS FACILITATE IMMUNE CELL ACTIVATION IN SEDENTARY, BUT NOT EXERCISE-TRAINED, RATS[1,2]

## 3.1 INTRODUCTION

Components of both the innate (natural) and acquired (specific) immune systems are important contributors to host defense against invading pathogens and spontaneously arising tumour cells (Whiteside and Herberman 1995, Robins 1986). T lymphocytes, part of the specific cell-mediated immune system, can be activated by interaction with different stimuli such as antigens and mitogenic lectins. Activated T helper ($CD4^+$) cells can mature into a subset of $CD4^+$ cells (Th1), which release interleukin-2 (IL-2), resulting in further activation and proliferation of T cells and expression of the IL-2 receptor (Cantrell and Smith 1984) and transferrin receptor (Neckers and Cossman 1983) on the cell surface. The appearance of both types of receptor is critical for the subsequent proliferation of activated T cells (Neckers and Cossman 1983). In addition, IL-2 upregulates activation of cytotoxic natural killer (NK) cells (Britten et al. 1984) and macrophages (Taub and Cox 1995). Immune activation of $CD8^+$ T cells can generate a population of effector cells with lytic capability called cytotoxic T lymphocytes which have important roles in the recognition and destruction of altered self-cells (Robins 1986).

In experimental cancer models, feeding high levels of fish oil-derived long-chain n-3 fatty acids, eicosapentaenoic acid (C20:5n-3, EPA) and docosahexaenoic acid (C22:6n-3, DHA), inhibits tumour growth (Karmali et al. 1984) and low to moderate-intensity exercise increases host resistance to tumourigenesis (Baracos 1989). Although it has been suggested that these effects occur via alterations in the host immune system, their precise cellular and molecular mechanisms are not known. Furthermore, the combined impact of dietary n-3 fatty acids and exercise on immunocompetence has not been assessed.

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[1] A version of this chapter has been published. Robinson, L. E. and Field, C. J. (1998) Journal of Nutrition 128: 498-504.

[2] This work was presented in part at Canadian Federation of Biological Societies, June 1996, London, Ontario and published in part in abstract form (Robinson, L. E., Goruk, S. D., and Field, C. J. High n-3 fatty acid diet reduces exercise-stimulated immune response. CFBS 39:A389).

There is evidence from both human and animal studies that diets rich in EPA and DHA can affect immune cell activation in both the innate and specific immune systems (Jolly et al. 1997, Yaqoob and Calder 1993, Meydani et al. 1991). For example, animals fed high levels of fish oil have suppressed NK cell cytotoxic activity (Sanderson et al. 1995, Yaqoob et al. 1994a) and T lymphocyte proliferation in response to mitogen (Sanderson et al. 1995). In mice, feeding highly purified EPA or DHA ethyl esters has also been shown to affect the process of immune cell activation by suppressing T cell IL-2 secretion and subsequent proliferation *in vitro* (Jolly et al. 1997). However, the physiological relevance of these effects is not clear, since many of these studies involved diets that were deficient in the essential n-6 fatty acid linoleic acid (18:2n-6, LA) and that contained levels of n-3 fatty acids far above those consumed by most humans. The specific dietary balance of long-chain n-3 fatty acids (EPA and DHA) and n-6 fatty acids necessary to promote immunocompetence has not been established.

Both human and animal studies have shown that exercise-induced immunomodulation may occur through an alteration in the types of immune cells present in different lymphoid organs and functional changes at the cellular level (Hoffman-Goetz and Pedersen 1994, Nieman and Nehlsen-Cannarella 1994). For example, exercise changes the proportion of different immune cells within blood (Field et al. 1991), decreases lymphocyte proliferation (Tvede et al. 1989), and increases NK cell cytotoxicity (Nieman et al. 1993). Compared with the number of studies investigating chronic exercise-training (involving bouts of high-intensity exercise) which have been associated with both deleterious and beneficial effects on immunocompetence (Hoffman-Goetz and Pedersen 1994, Lin et al. 1993), fewer studies have reported on the immunomodulatory effects of low- to moderate-intensity exercise-training, such as that experienced during regular non-exhaustive exercise.

Using a low-intensity exercise-training protocol in rats, Shewchuk et al. (1997a) demonstrated that non-exhaustive swim-training increased T cell mitogenic response, suggesting enhanced cell-mediated immune function. Although lymphocyte proliferation studies provide indirect evidence of immune cell activation, there is little direct evidence on the effect of exercise on the expression of cell surface markers of immune activation, such as the transferrin receptor (CD71). Also, since the majority of exercise immunology studies have been conducted using immune cells from blood, little is known about the effect of exercise-training on other lymphoid organs. Furthermore, many exercise studies have not controlled for dietary fat, a factor which modulates immune function.

In animal studies, exercise and diet can be precisely controlled so as to define relationships between diet, exercise, and components of the immune system which are inaccessible in humans, such as spleen. The objectives of this study were to determine the individual and combined effects of dietary long-chain n-3 fatty acids and low-intensity exercise (swim-training) on immunocompetence in healthy rats.

## 3.2 MATERIALS AND METHODS

**Animals, diets, and exercise.** Experiments were reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Twenty-eight male (152 ± 7 g) and 28 female (108 ± 3 g) adult Fischer 344 rats (Charles River Laboratories, St. Constant, QB, Canada) were housed individually in plastic shoebox cages in a temperature controlled room (23 °C) maintained on a 12 h light/dark cycle. Body weight and food intake were recorded every third day throughout the study.

Rats were randomly assigned to be fed one of two nutritionally complete, semi-purified diets (Teklad Test Diets, Madison, WI) containing (per kg) 270 g high protein casein, 408 g carbohydrate (200 g starch; 208 g dextrose), and 200 g fat. The diets were supplemented with the following (per kg): 1.4 g choline, 6.2 g inositol, 2.5 g of L-methionine, 10 g A.O.A.C. (Association of Official Analytical Chemists) vitamin mix, 51 g Bernhart-Tomerelli mineral mix, and 50 g non-nutritive cellulose. The composition of the mineral and vitamin mix has been previously reported (Clandinin and Yamashiro 1980). Both experimental diets provided a polyunsaturated to saturated fatty acid ratio (P/S) of 1, as determined by gas-liquid chromatography (Field et al. 1988). The fatty acid composition of the diets is presented in **Table 3.1**. The two diets differed only in the composition of polyunsaturated fat, providing two different levels of long-chain n-3 fatty acids from a mixed fish oil source (P-28 Nisshin lot # 28020, Nisshin Flour Milling Co., Ltd., Tokyo, Japan): low (0 g/kg) or high (51 g/kg of total fat). The only source of n-3 fatty acids in the low n-3 diet was α-linolenic acid (C18:3n-3; LNA, 12 g/kg of total fat), provided by linseed oil (Galaxy Enterprises Ltd., Edmonton, AB, Canada). The high n-3 diet contained both LNA (2 g/kg of total fat) and long-chain n-3 fatty acids provided by fish oil (per kg of total fat): 32 g C20:5n-3, 2 g C22:5n-3, and 8 g C22:6n-3. All animals were given free access to food and water for 4 wk.

At the start of the study, rats in each diet group were randomly assigned to one of two activity groups. Rats either remained sedentary or followed a low-intensity swimming program for 4 wk, training progressively to swim a maximum of 3 h/d (6 d/wk) as previously described (Baracos 1989). To control for the animals' response to water, rats in the sedentary group were subjected to an identical period of water exposure by standing daily in 10 cm of water (33-35 °C). Exercise was performed at the end of the dark cycle.

Rats were food-deprived overnight prior to being killed. On experiment days, rats swam for 2 h (or remained sedentary) and were subsequently killed by $CO_2$ asphyxiation and cervical dislocation. All assays were performed on individual rats (*n* = 14/treatment group unless otherwise indicated) with the exception of the NK cell cytotoxicity assay (sedentary, *n* = 12/diet; exercise-trained, *n* = 5/diet).

**Splenocyte isolation.** Following $CO_2$ asphyxiation and cervical dislocation, the spleen from each animal was removed aseptically, weighed, and placed in a petri dish containing cold sterile-filtered Krebs-Ringer-N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) buffer (KRH, pH 7.4, Appendix A) supplemented with bovine serum albumin (BSA, 5g/L; Sigma Chemical, St. Louis, MO) and 1% (v/v) antimycotic-antibiotic solution ($1 \times 10^5$ U/L penicillin, 100 mg/L streptomycin, and 25 mg/L amphotericin B; Gibco BRL, Burlington, ON, Canada). Under sterile conditions, spleens were pressed through 100 μm mesh nylon to isolate splenocytes (Wu et al. 1991). The resulting cell suspension was centrifuged at 228 g (Beckman J2-HC, Beckman Instruments, Palo Alto, CA) for 10 min at 4°C and the cell pellet was resuspended in 1.2 mL of lysis buffer (see Appendix A for composition) for 3 min on ice to lyse the red blood cells. After exactly 3 min, the cell suspension was diluted in 30 mL of sterile KRH (pH 7.4) supplemented with BSA (5 g/L) and splenocytes were washed two times. Subsequently, the supernatant was removed and splenocytes were resuspended in 10 mL of complete culture media [CCM; RPMI 1640 (Fisher Scientific, Edmonton, AB, Canada) supplemented with 4% (v/v) heat-inactivated fetal calf serum (FCS; ICN, Montréal, QB, Canada), 1% (v/v) antimycotic-antibiotic solution, glutamine (4 mmol/L; Gibco BRL), HEPES (25 mmol/L; Gibco BRL), and 2-mercaptoethanol (2.5 µmol/L; Sigma Chemical)]. Viability was determined by trypan blue exclusion (Sigma Chemical).

**Splenocyte activation.** Isolated splenocytes ($2.5 \times 10^9$ cells/L) in CCM were incubated in 25 $cm^2$ sterile tissue culture flasks (Corning Glass Works, Corning, NY) for 48 h in a humidified atmosphere at 37 °C in the presence of 5% $CO_2$. The cell culture media contained either no mitogen (unstimulated cells) or was supplemented with Concanavalin A (Con A), a polyclonal T lymphocyte mitogen (5 mg/L; ICN). After 48 h, cells were centrifuged at 228 g (Beckman J2-HC) for 10 min at 4°C. The pelleted cells were then washed twice in phosphate buffered saline (PBS, Appendix A) supplemented with BSA (20 g/L). Indirect single- and double-label immunofluorescence analyses (described below) were then performed on both unstimulated and stimulated splenocytes.

**Indirect immunofluorescence (phenotype) assay.** Lymphocyte subsets from freshly isolated splenocytes and cultured splenocytes (unstimulated and stimulated) were identified by indirect immunofluorescence assay (Field 1995) using supernatants from hybridomas secreting mouse monoclonal antibodies (mAb) specific for the different rat mononuclear cell subsets (Barclay et al. 1993). The following mAb were used: OX19 (CD5), which recognizes a glycoprotein on the surface of thymocytes and peripheral T lymphocytes; w3/25 (CD4), which reacts with a glycoprotein on rat T helper lymphocytes and peritoneal macrophages; OX8 (CD8a), which recognizes thymocytes, T suppressor/cytotoxic lymphocytes and NK cells; OX12, which recognizes a determinant on the rat κ chain of immunoglobulin (Ig) on B lymphocytes; OX42 (CD11b/c), which reacts with a receptor found on most monocytes and macrophages, granulocytes and dendritic cells; 3.2.3 (CD161), which recognizes rNKR-P1A on rat NK cells and a subset of T cells; w3/13, which recognizes pan T-cells, polymorphs, plasma cells, and NK cells and OX26 (CD71), which recognizes the transferrin receptor on activated T and B cells, and macrophages. The mAb used in this assay are defined in **Table 3.2.** All mAb were kindly provided by Dr. A. Rabinovitch (University of Alberta, Edmonton, AB, Canada) with the exceptions of 3.2.3, w3/13, and OX26 (Cedarlane Laboratories Ltd., Hornby, ON, Canada). All mAb were mouse anti-rat IgG.

Non-sterile 96-well V-bottom microtiter plates (Fisher Scientific) were preconditioned with PBS containing FCS (40 g/L) at room temperature for at least 20 min. Then, freshly isolated splenocytes and cultured splenocytes were aliquoted at a concentration of 2-5 x $10^5$ cells/well. The cell suspension was then centrifuged at 200 g, 4°C (Jouan Centrifuge, Jouan Inc, Winchester, VA) for 1 min, the supernatant was aspirated, and the microtiter plate was vortexed to break up cell pellets. For indirect single-label (one colour) phenotype analysis, cells were then incubated for 30 min at 4 °C with an aliquot of the primary antibody (OX19, w3/25, OX8, OX12, OX42, w3/13, or 3.2.3), washed three times in 200 µL of PBS containing FCS (40 g/L), and incubated for another 30 min at 4 °C with 50 µL of a 1:300 dilution of fluorescein isothiocyanate-conjugated goat anti-mouse IgG (FITC, Organon Teknika Inc., Scarborough, ON, Canada) that has no cross reaction to rat IgG. To determine background fluorescence due to non-specific binding of FITC, samples were incubated for 30 min at 4 °C with FITC alone.

The functional state of immune cells can be measured by performing an indirect double-label (two colour) immunofluorescence assay using a mAb against the transferrin receptor (CD71), a marker of cellular activation (Neckers and Cossman 1983), plus various mAb identifying lymphocyte subpopulations. For this assay, the phenotypic antibody (OX19, w3/25, OX8, OX12, or OX42) was incubated with FITC as described above. Following FITC incubation and washing, an aliquot of the activation marker antibody (OX26) was added to each well and incubated for 30 min at 4 °C. Cells were then washed three times (as described above) prior to incubation for 30 min at 4 °C with 10 µL of a 1:25 dilution of phycoerythrin-conjugated goat anti-mouse IgG (Cedarlane

Laboratories Ltd). As with FITC, samples were incubated for 30 min at 4 °C with phycoerythrin alone to determine background fluorescence due to non-specific binding. Finally, the cells were washed 3 times (as described above), fixed in 200 µL of PBS containing paraformaldehyde (10 g/L; Anachemia Science, Montréal, QB, Canada), and relative fluorescence intensities for each antibody were determined by flow cytometry (FACScan, Becton Dickinson, Sunnyvale, CA). Unwanted events (dead cells and debris) were detected on the basis of forward scatter and side scatter and were excluded from subsequent phenotype analyses by electronic gating of the viable splenocyte population. Ten-thousand viable events were collected in list mode and all subsequent immunofluorescence analyses were performed on only these cells using Lysis II Software (Becton Dickinson). The resulting percentages were corrected for background fluorescence using the analysis of cells incubated with FITC or phycoerythrin alone.

**NK cell cytotoxicity assay.** A 4 h sodium chromate ($^{51}$Cr) release assay was performed using NK cell-sensitive YAC-1 cells (American Type Culture Collection, Rockville, MD) as targets and freshly isolated splenocytes as effector cells (Field 1995). YAC-1 cells were spun for 10 min at 228 g (Beckman J2-HC), resuspended in 1 mL of 100% FCS and counted for viability using trypan blue exclusion. Target cells suspended in RPMI 1640 supplemented with 20% (v/v) FCS, 1% (v/v) antimycotic-antibiotic solution, glutamine (4 mmol/L) and HEPES (25 mmol/L) were labeled with 500 µCi of $^{51}$Cr (5.55 MBq, Amersham, Oakville, ON, Canada) and incubated for 90 min at 37°C in a gently shaking water bath. After 90 min, the $^{51}$Cr-labeled YAC-1 cells were gently washed 3 times to remove extracellular radioactivity. Each wash consisted of spinning the cells for 10 min at 228 g removing the supernatant and resuspending in 10 mL CCM with 10% FCS (v/v). In the final wash, cells were resuspended in 0.5 mL CCM with 20% FCS (v/v) and counted again for viability using trypan blue exclusion. Cells were then diluted to 2 x $10^5$ cells/mL in CCM with 20% FCS (v/v). Fifty µL of $^{51}$Cr-labeled target cells and 100 µL of effector cells were added in triplicate at different effector:target ratios (5:1 to 100:1) to wells of 96-well V-bottom microtiter plates (Costar®, Cambridge, MA). Plates were briefly spun until 228 g was reached and the centrifuge was then braked. Plates were incubated for 4 h at 37°C in a humidified 5% $CO_2$ atmosphere. After incubation, plates were centrifuged at 200 g for 10 min and the amount of $^{51}Cr$ released was determined by counting a 75 µL aliquot of supernatant from each well in a gamma counter (Beckman gamma 8000, Beckman Instruments, Inc., Mississauga, ON, Canada) to determine the extent of target cell lysis. Spontaneous $^{51}Cr$ release was determined by incubation of labeled target cells alone. Maximum release was determined by incubation of labeled targets with a detergent, 4% (v/v) Triton-X 100 (BDH Chemicals, Toronto, ON, Canada) in CCM with 10% FCS (v/v). The percentage lysis of the target cells was calculated using the following formula: Specific lysis (%) = 100 x [mean experimental $^{51}Cr$ release (cpm)-mean spontaneous $^{51}Cr$ release (cpm)] / [mean maximum $^{51}Cr$ release (cpm)-mean spontaneous $^{51}Cr$ release (cpm)]. Results were also calculated on a per cell basis using the number of NK cells present in the effector cell samples, as determined by indirect immunofluorescence assay (described above) using the 3.2.3 (anti-CD161/NK cell) mAb. This was expressed as lytic units with one lytic unit being equal to the number of effector cells (x $10^{-3}$) required to cause 20% lysis of target cells (effector:target cell ratios of 5:1, 25:1, 50:1 and 100:1 were studied).

**Fatty acid analysis.** Lipids were extracted from splenocytes by a modified Folch (Folch et al. 1957) procedure (Field et al. 1988). Individual phospholipids were separated from the lower phase on thin layer chromatography plates (HPK silica gel 60 Å 10 x 10 cm, Whatman, Clifton, NJ) using the following solvent system: chloroform:methanol:2-propanol:0.25% (w/v) KCl:triethylamine (30:9:25:6:18 by volume) as previously described (Touchstone et al. 1980). Separated phospholipids were visualized with 8-anilino-1-naphthalene-sulfonic acid (Sigma Chemical) and identified under ultraviolet light with appropriate standards (Sigma Chemical). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) fatty acid methyl esters were prepared from the scraped silica band using 14% (w/v) $BF_3$/methanol reagent (Morrison and Smith 1964) and separated by automated gas liquid chromatography (Vista 6010, Varian Instruments, Georgetown, ON). Chromatography was performed using a fused silica BP20 capillary column (25 m x 0.25 mm internal diameter, Varian Instruments) as previously described (Field et al. 1988). The analytical conditions used separate all saturated, mono-, di-, and polyunsaturated fatty acids from C14 to C24 carbons in chain length.

**Statistical analysis.** Results are presented as means ± SEM. All statistical analyses were conducted using the SAS statistical package (Version 6.11, SAS Institute, Cary, NC). The effect of sex was determined by three-way analysis of variance procedure (ANOVA). If no effect of sex was found, the main effects (diet and exercise) were analyzed by two-way ANOVA. Significant ($p < 0.05$) differences between treatment groups were identified by least squares means. NK cell cytotoxicity, food intake, and body weight were compared among groups by a two-way split-plot (repeated measures) ANOVA (Steel and Torrie 1980). For phenotype data, a paired *t* test was used to compare freshly isolated splenocytes to those cultured without Con A for 48 h (significant effect of culture, $p < 0.05$). Similarly, a paired *t* test was used to compare splenocytes cultured without Con A for 48 h to those cultured with Con A for 48 h (significant effect of stimulation, $p < 0.05$).

## 3.3 RESULTS

**Animal characteristics.** Diet did not significantly affect food intake, final body weight, weight change, spleen weight, or the number of spleen cells isolated per gram of spleen (**Table 3.3**); therefore, rats in the low and high n-3 diet groups within each activity group were combined for statistical analysis to examine the effect of exercise (Table 3.3). Exercise-training did not significantly affect average daily food intake or final body weight in male or female rats (Table 3.3). Sedentary males gained significantly ($p < 0.01$) more weight than exercise-trained males, while weight gain did not differ between sedentary and exercise-trained females (Table 3.3). For both male and female rats, spleen weight ($p < 0.0001$) and spleen weight per gram of body weight ($p < 0.0001$) were significantly lower in the exercise-trained group (Table 3.3). However, the number of immune cells ($\times 10^6$) isolated per gram of spleen (377 ± 12, $n = 56$) did not differ by sex or activity (Table 3.3).

**Single-label phenotypes in fresh and cultured (with and without mitogen) splenocytes.** As there was no significant effect of sex of the rat on immune variables in this study, male and female rats were combined within each treatment group. Neither diet nor exercise-training significantly affected the proportion (% of total) of lymphocyte subsets in spleen (**Tables 3.4-3.6**). However, the CD4/CD8 ratio in freshly isolated splenocytes was significantly lower ($p < 0.01$) when exercise-trained rats were fed the high n-3 diet compared with the low n-3 diet (Table 3.4). Treatment groups were combined within each cell culture condition to examine changes in immune phenotypes following culture and mitogen stimulation **(Table 3.7)**. The relative percentages of CD4$^+$ T helper cells, B cells and macrophages were significantly higher in splenocytes cultured without Con A for 48 h than in freshly isolated splenocytes (Table 3.7). The relative percentages of total T cells (CD5$^+$ and w3/13$^+$) were significantly lower in splenocytes cultured with Con A for 48 h than in those cultured without mitogen, while the relative percentages of T suppressor/cytotoxic cells (CD8$^+$) and macrophages were significantly higher after culture with Con A (Table 3.7).

**Double-label phenotypes in fresh and cultured (without mitogen) splenocytes.** For freshly isolated splenocytes and those cultured for 48 h without mitogen, neither diet nor exercise-training significantly affected the proportion (% of total) of CD71$^+$ (transferrin receptor positive) splenocytes identified as CD5$^+$, CD4$^+$, CD8$^+$, or macrophages (**Tables 3.8-3.9**). Therefore, treatment groups were combined within each cell culture condition for double-label phenotypes **(Table 3.10)**. The relative percentage of CD71$^+$ macrophages was significantly lower in splenocytes cultured without Con A for 48 h than in freshly isolated splenocytes (Table 3.10), while the relative percentages of CD71$^+$ CD5$^+$ T cells, CD71$^+$ CD4$^+$ T helper cells, and CD71$^+$ CD8$^+$ T suppressor/cytotoxic cells were significantly increased following culture without Con A for 48 h (Table 3.10).

**Double-label phenotypes in cultured (with mitogen) splenocytes.** Double-label phenotyping analysis of splenocytes following 48 h stimulation with Con A showed that, in the sedentary group, rats fed the high n-3 diet had a significantly higher proportion (% of total cells) of CD5$^{+}$, CD4$^{+}$, CD8$^{+}$, B cells and macrophages that were CD71$^{+}$ compared with low n-3-fed rats (**Figure 3.1**). However, exercise-trained rats fed the high n-3 diet had a significantly lower proportion of CD4$^{+}$ and B cells that were CD71$^{+}$ after Con A stimulation compared with low n-3-fed rats (Figure 3.1). In the low n-3 diet group, rats that were exercise-trained had a significantly higher proportion of CD5$^{+}$, CD4$^{+}$ and B cells that were CD71$^{+}$ after stimulation with Con A than did sedentary rats. However, in the high n-3 diet group, rats that were exercise-trained rats had a significantly lower proportion of CD4$^{+}$, CD8$^{+}$ and B cells that were CD71$^{+}$ after Con A stimulation compared with sedentary rats (Figure 3.1).

**NK cell cytotoxicity**. There was a significant effect of diet, activity, and diet by activity interaction on NK cell cytotoxic activity as determined by a two-way split-plot (repeated measures) ANOVA (**Figure 3.2**). At the 5:1 and 25:1 effector:target cell ratios, sedentary rats fed the high n-3 diet had a higher ($p < 0.02$) % specific lysis of target YAC-1 cells (5.1 ± 0.6%; 13 ± 1%, $n = 12$) relative to sedentary rats fed the low n-3 diet (3.2 ± 0.3%; 10 ± 1%, $n = 11$) (Figure 3.2). There was no effect of diet on NK cell cytotoxicity at the 5:1 and 25:1 ratios when rats were exercise-trained (Figure 3.2). For cells from sedentary rats, at the 50:1 and 100:1 ratios those fed the high n-3 diet had higher ($p < 0.001$) NK cell cytotoxic activity against the target cells compared with those fed the low n-3 diet (**Figure 3.3**). For exercise-trained rats, dietary n-3 fatty acids did not significantly affect NK cell activity (Figure 3.3). When rats were fed the low n-3 diet there was no effect of exercise-training on NK cell cytotoxicity (Figure 3.3). However, when rats were fed the high n-3 diet, exercise-trained rats had lower ($p < 0.001$) NK cell cytotoxic activity compared with sedentary rats (Figure 3.3).

Sedentary rats fed the high n-3 diet had lower ($p < 0.05$) lytic units (the number of effector cells (x $10^{-3}$) required to cause 20% lysis of YAC-1 cells) than sedentary rats fed the low n-3 diet (**Figure 3.4**). Diet did not significantly affect lytic units for cells from exercise-trained rats (Figure 3.4). When rats were fed the low n-3 diet, exercise-training did not significantly affect lytic units (Figure 3.4). However, in the high n-3 diet group, exercise-trained rats had higher ($p < 0.05$) lytic units compared with sedentary rats (Figure 3.4).

**Fatty acid composition of splenocytes.** Sex of the rat did not significantly affect the fatty acid composition of splenocytes; therefore, male and female rats were combined within each diet group. Splenocytes from both sedentary and exercise-trained rats fed the high n-3 diet had a significantly higher total n-3 content [20:5(3), 22:5(3), 22:6(3)] and lower n-6/n-3 fatty acid ratio in PC, PE, and PI (**Tables 3.11-3.13**). In both the low and high n-3 diet groups, exercise-training did not significantly affect the total n-3 or n-6 fatty acid content of PC (Table 3.11). However, in the low n-3 diet group, exercise-trained rats had a significantly lower n-6/n-3 ratio in PC relative to sedentary rats (Table 3.11). More specifically, in the low n-3 diet group, exercise-trained rats had a significantly lower % of C20:4(6) and C22:4(6) and a higher % of C20:5(3) in PC compared with sedentary rats (Table 3.11). However, the same fatty acids, as well as the n-6/n-3 ratio in PC, were not significantly affected by exercise-training when rats were fed the high n-3 diet (Table 3.11). The total n-6 and n-3 fatty acid composition of PE was not significantly affected by exercise-training when rats were fed the low n-3 diet, although the P/S ratio was significantly lower in exercise-trained rats fed the low n-3 diet compared with sedentary rats fed the same diet (Table 3.12). Also, in the high n-3 diet group, exercise-trained rats had a significantly higher % of C22:6(3) and total n-3 content in PE relative to sedentary rats (Table 3.12). In PI, exercise-training significantly increased the % of C18:3(3) and C18:4(3) in both the low and high n-3 diet groups (Table 3.13). In addition, exercise-training significantly decreased the % of C22:4(6) in PI, but only in the low n-3 diet group, while the % of C22:5(6) was significantly increased by exercise-training, but only in the high n-3 diet group (Table 3.13). Exercise-training did not significantly affect total saturated or monounsaturated fatty acids in PC, PE or PI (Tables 3.11-3.13).

## 3.4 DISCUSSION

In experimental cancer, feeding high levels of fish oil-derived long-chain n-3 fatty acids, EPA and DHA, inhibits tumour growth (Karmali et al. 1984), as does regular exercise of low- to moderate-intensity (Baracos 1989). The mechanism for these inhibitory effects is not known, but may involve alterations in the host immune system. This is the first study to assess the combined impact of dietary long-chain n-3 fatty acids and low-intensity swim-training on immunocompetence in healthy rats. Our major finding is that feeding a semi-purified diet (P/S = 1) containing approximately 5% (w/w) of the total fat as long-chain n-3 fatty acids facilitates immune cell activation in sedentary, but not exercise-trained, rats.

It has been hypothesized that diet and exercise-induced alterations in immunity might be determined by assessing changes in the proportion of cells within lymphocyte subsets (T and B cells, NK cells, and macrophages) (Hoffman-Goetz and Pedersen 1994). In agreement with previous studies (Jeffery et al. 1996, Yaqoob et al. 1994b, Payan et al. 1986), long-chain n-3 fatty acids did not significantly alter the distribution of immune cell types present in spleen (Table 3.4). Despite no effect of diet and exercise, culturing splenocytes altered the proportion of immune cell types (Table 3.7). As expected, the proportion of macrophages was significantly increased in both the unstimulated and stimulated condition (Table 3.7). Surprisingly, the proportion of macrophages that were activated (transferrin receptor positive, CD71$^+$) following culture without mitogen was decreased (Table 3.10). This may have been due to the adherence of activated macrophages to the plates during culture as it is difficult to recover activated cells that represent less than 10% of the total cell population.

Immune status can be monitored by measuring the activation state of immune cells. Cell activation is a complex process which involves a number of plasma membrane-associated events, including activation of phospholipase C (with generation of the second messengers 1,4,5-inositol triphosphate and diacylglycerol), $Ca^{2+}$ mobilization, and activation of protein kinase C (Hadden 1988). These processes ultimately result in proliferation, target cell lysis, production of cytokines, and the expression of immune cell activation markers such as the transferrin receptor (CD71) (Neckers and Cossman 1983).

*In vitro*, the functional state of immune cells can be assessed by measuring the expression of CD71, after lymphocytes have been stimulated with a mitogen, such as Con A (Neckers and Cossman 1983). Compared with the number of studies investigating the effect of n-3 fatty acids on lymphocyte proliferation (Yaqoob and Calder 1993), fewer studies have provided evidence on the effect of EPA and DHA on the expression of CD71. In an *in vitro* study, Calder and Newsholme (1992) showed that CD71 expresssion on the surface of Con A-stimulated rat lymphocytes was inhibited by culture with polyunsaturated fatty acids. In another study, Yaqoob et al. (1994b) demonstrated that feeding rats a diet containing very high levels of fish oil had no effect on the proportion of CD71$^+$ cells following Con A stimulation of rat splenocytes. In contrast, we found that feeding dietary long-chain n-3 fatty acids to sedentary rats resulted in a higher proportion of T- and B-cells and macrophages that were activated (CD71$^+$) after Con A stimulation (Figure 3.1), suggesting an upregulation of the cell-mediated immune response. These results may be of importance because activated T helper (CD4$^+$) cells produce IL-2, which induces activation of NK cells, an important anticancer defense mechanism (Whiteside and Herberman 1995, Britten et al. 1984). In the present study, the cytotoxic activity of splenocytes was significantly higher for sedentary rats fed the high n-3 diet compared with the other three groups (Figures 3.2-3.3). Since the percentage of NK (3.2.3$^+$) cells present in the splenocyte population was not affected by diet, the increased cytotoxicity in high n-3-fed sedentary rats can be hypothesized to be due to a functional enhancement at the cellular level (lower lytic units, Figure 3.4). These findings contradict many previous reports in the literature (Sanderson et al. 1995, Yaqoob et al. 1994a, Fritsche and Johnstone 1990, Meydani et al. 1988). For example, Yaqoob et al. (1994a), reported that rats fed a diet containing 100% of fat from fish oil had significantly decreased NK cell activity. However, other studies indicate that this result may be a product of feeding extremely high n-3 levels, since feeding 10-12 g/100 g of total fat in the diet as long-chain n-3 fatty acids significantly increased NK cell cytotoxicity (Brouard and Pascaud 1993). The level of dietary long-chain n-3 fatty acids used by Brouard and Pascaud (1993) was approximately two times the level in our experimental diet, but was substantially lower than earlier studies which fed diets containing fish oil as the only fat source. Thus, it appears that very high levels of fish oil-derived EPA and DHA, which would be difficult to achieve in the human diet, may be inhibitory (Sanderson et al. 1995, Yaqoob et al. 1994a, Fritsche and Johnstone 1990, Meydani et al. 1988), while lower, more physiological, levels of long-chain n-3 fatty acids may be stimulatory in terms of NK cell cytotoxicity.

Exercise has also been shown to modulate immune function (Hoffman-Goetz and Pedersen 1994). For example, changes in the proportion of cells within immune cell subsets in peripheral circulation (Hoffman-Goetz and Pedersen 1994), but not spleen (Lin et al. 1993), have been related to exercise intensity and duration. High-intensity exercise is associated with transient increases in the absolute number and relative proportion of NK, B, and CD8$^+$ T-suppressor/cytotoxic cells and decreases in CD4$^+$ T helper cells in human blood (Field et al. 1991). Less is known about the effect of low-intensity exercise-training on immune phenotypes. Consistent with previous work using an identical swimming protocol (Shewchuk et al. 1997b), the proportion of splenocytes within lymphocyte subsets was not affected when exercise-trained rats were killed immediately post-exercise (Table 3.4).

In rats fed the low n-3 diet, exercise resulted in an increased proportion of T- and B-cells that were activated (CD71$^+$) after Con A stimulation (Figure 3.1), an observation indicative of immune enhancement. The expression of the transferrin receptor on the cell surface is crucial for the subsequent proliferation of activated T cells (Neckers and Cossman 1983). A previous study showed that swim-training increases the proliferative response of T cells to Con A stimulation (Shewchuk et al. 1997a), providing further support for our finding that cell-mediated immune function is enhanced immediately post-exercise in swim-trained rats. Interestingly, immune activation following exercise may depend, in part, on the type of fat in the diet since the proportion of immune cells that were activated (CD71$^+$) following Con A stimulation was suppressed when exercise-trained rats were fed long-chain n-3 fatty acids (Figure 3.1). These findings suggest the importance of controlling dietary fat in studies investigating the role of exercise in modulating immune cell activation in healthy rats.

This study confirms the previous finding that low-intensity exercise-training does not significantly affect NK cell cytotoxic activity when rats are fed diets without long-chain n-3 fatty acids (Figures 3.2-3.3) (Shewchuk et al. 1997b). However, surprisingly, exercise resulted in suppressed NK cell activity when rats were fed EPA and DHA (Figure 3.2-3.3). Again, our results suggest that the ability of exercise to alter immune function in rats may be modified by the type of fat in the diet.

The effect of n-3 fatty acids and exercise-training on the immune system may involve modulation of membrane-mediated functions through changes in membrane lipid composition (Calder 1998). It is well established that changes in dietary fat composition can induce significant alterations in the composition and function of membranes in a variety of human and animal tissues (Field et al. 1988, Clandinin et al. 1985). In lymphocytes, membrane-associated events play a pivotal role in signal transduction (Hosack-Fowler et al. 1993), the expression of surface markers (Jenski et al. 1995, Yaqoob et al. 1994b) and cellular activation (Calder et al. 1994, Hadden 1988), all of which are important in immune cell function. Also, many functions of the immune system depend on interactions between effector and target cell membranes. Thus, changing the membrane composition of such cells may influence immune responses. This study confirms previous findings that dietary fat significantly alters the fatty acid composition of immune cell phospholipids (Field et al. 2000, Hosack-Fowler et al. 1993). Exercise-training has also been shown to alter the fatty acid composition of tissues, such as erythrocytes (Sumikawa et al. 1993, Ågren et al. 1991) and skeletal muscle (Helge et al. 1999, Andersson et al. 1998). However, the effect of exercise-training on immune cell membrane fatty acid composition has not been as widely studied. Although the effects of exercise-training on splenocyte membrane composition were limited, this is the first report of a significant interaction between dietary long-chain n-3 fatty acids and exercise-training on the fatty acid composition of PC, PE and PI in immune cells (Tables 3.11-3.13). For example, in low n-3-fed rats, exercise-training decreased the n-6/n-3 ratio in PC, while in high n-3-fed rats exercise-training increased total n-3 fatty acids in PE. In the present study, feeding long-chain n-3 fatty acids in the diet altered the fatty acid composition of immune cells which, in turn, may have altered important membrane-dependent properties, such as signal transduction pathways (Hosack-Fowler et al. 1993), thereby facilitating immune cell activation and NK cell cytotoxicity. Furthermore, since exercise-training altered the fatty acid composition of splenocyte phospholipids, it is possible that the observed effects of exercise on immune cell activation (increased CD71 expresssion) may also occur via membrane-associated events. However, this requires further study.

Although we found that dietary long-chain n-3 fatty acids and exercise-training were immunostimulatory on an individual basis, the combination of dietary n-3 fatty acids and low to moderate-intensity exercise-training did not enhance the immune parameters assayed. Thus, our results suggest that the immune responses produced by the high n-3 diet and exercise-training are not synergistic. To our knowledge, this is the first report of the interaction between dietary n-3 fatty acids and exercise-training with respect to immune cell membrane composition and immunocompetence. The mechanism underlying the interaction between dietary long-chain n-3 fatty acids and exercise-training requires further investigation.

This study has shown that providing long-chain n-3 fatty acids in the diet at a level achievable by segments of the human population, modulates immune cell composition and function. Critically, however, the immune response to n-3 polyunsaturated fatty acids depends on the level of physical activity: immunostimulatory effects of long-chain n-3 fatty acids are observed in sedentary, but not exercise-trained, rats. These findings may help to explain current controversies in exercise immunology literature, since diet is infrequently controlled in exercise studies. Furthermore, since there is an increasing amount of evidence implicating both dietary long-chain n-3 fatty acids and regular, low-intensity exercise as playing beneficial roles in cancer prevention and treatment, our findings may be important when developing clinical strategies for cancer treatment.

**TABLE 3.1 Fatty Acid Composition of Low and High n-3 Diets Fed to Sedentary and Exercise-Trained Rats for 4 wk[1]**

| | Low n-3 Diet | High n-3 Diet |
|---|---|---|
| Fatty Acid | % w/w | |
| C14:0 | 1.3 | 2.0 |
| C16:0 | 15.7 | 15.7 |
| C18:0 | 26.9 | 25.5 |
| C18:1 (9) | 8.4 | 8.2 |
| C18:2 (6) | 44.3 | 39.3 |
| C18:3 (3) | 1.2 | 0.2 |
| C20:5 (3) | nd | 3.2 |
| C22:5 (3) | nd | 0.2 |
| C22:6 (3) | nd | 0.8 |
| SFA | 45.6 | 44.8 |
| MUFA | 8.7 | 10.4 |
| PUFA | 45.3 | 44.5 |
| n-6 PUFA | 44.3 | 39.6 |
| n-3 PUFA | 1.2 | 5.1 |
| n-6/n-3 ratio | 37 | 7.8 |
| P/S ratio | 1.0 | 1.0 |

[1] Values are g/100 g of total fat, except for ratios. Diets contained 200 g/kg of fat from a mixture of sources [safflower oil, hard beef tallow, linseed oil (low n-3 diet only), and fish oil (high n-3 diet only]. Minor fatty acids are not reported, therefore totals do not add up to 100%. Abbreviations used: nd, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids.

**TABLE 3.2 Monoclonal Antibodies Used in Indirect Immunofluorescence (Phenotype) Assay**

| Clone[1] | Specificity | Description |
|---|---|---|
| OX19 | CD5 | Reacts with 69 kDa surface glycoprotein on thymocytes and peripheral T cells |
| w3/13 | CD43 | Reacts with 95 kDa surface glycoprotein on thymocytes and peripheral T cells |
| w3/25 | CD4 | Reacts with 48-53 kDa surface glycoprotein on T helper cells and peritoneal macrophages |
| OX8 | CD8a | Reacts with 34 kDa α chain of CD8 antigen on thymocytes, T suppressor/cytotoxic cells, natural killer (NK cells), and some activated T helper cells |
| OX12 | Ig κ chain | Reacts with immunoglobulin (Ig) κ chain on B cells |
| OX42 | CD11b/c | Reacts with 160, 103, and 95 kDa cell surface proteins on most resident peritoneal and activated macrophages, granulocytes, monocytes, and dendritic cells |
| 3.2.3 | CD161 | Reacts with NKR-P1A on NK cells and subset of T cells |
| OX26 | CD71 | Reacts with transferrin receptor on activated T and B cells, and macrophages |

[1] All monoclonal antibodies were kindly provided by Dr. A. Rabinovitch (University of Alberta, Edmonton, AB, Canada) with the exceptions of w3/13, 3.2.3, and OX26 (Cedarlane Laboratories Ltd., Hornby, ON, Canada). All mAb were mouse anti-rat IgG.

**TABLE 3.3 Effect of Diet, Exercise-Training and Sex on Food Intake, Body Weight and Spleen Weight of Rats**[1]

| | Sedentary | | | | Exercise-Trained | | | | Significance, p < | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Low n-3 | High n-3 | Low n-3 | High n-3 | Low n-3 | High n-3 | Low n-3 | High n-3 | | |
| | M | M | F | F | M | M | F | F | Sex | Activity |
| Food Intake, g/kg body weight /d | 73 ± 2 | 70 ± 1 | 71 ± 1 | 71 ± 1 | 71 ± 2 | 73 ± 3 | 75 ± 2 | 78 ± 1 | NS | NS |
| Final Body Weight, g | 203 ± 14 | 204 ± 9 | 131 ± 5 | 134 ± 6 | 192 ± 5 | 196 ± 11 | 130 ± 4 | 131 ± 4 | 0.0001 | NS |
| Weight Increase, g | $56 \pm 4^a$ | $57 \pm 5^a$ | $23 \pm 4^c$ | $24 \pm 2^c$ | $38 \pm 5^b$ | $37 \pm 7^b$ | $20 \pm 3^c$ | $27 \pm 1^c$ | 0.0001 | 0.01 |
| Spleen Weight, mg | 429 ± 25 | 430 ± 21 | 335 ± 10 | 337 ± 12 | 334 ± 14 | 367 ± 23 | 295 ± 15 | 306 ± 14 | 0.0001 | 0.0001 |
| Spleen Weight, g/ kg body weight | 2.1 ± 0.1 | 2.1 ± 0.1 | 2.6 ± 0.1 | 2.5 ± 0.1 | 1.7 ± 0.4 | 1.9 ± 0.1 | 2.3 ± 0.1 | 2.3 ± 0.1 | 0.0001 | 0.0001 |
| Spleen Cells/g spleen ($\times 10^6$) | 415 ± 16 | 370 ± 44 | 404 ± 74 | 342 ± 12 | 314 ± 40 | 371 ± 49 | 372 ± 21 | 435 ± 17 | NS | NS |

[1] Values are means ± SEM ($n \geq 6$/group). Abbreviations used: M, male rats; F, female rats.

[2] There was no effect of diet by three-way ANOVA; therefore, low n-3- and high n-3-fed rats were combined within each activity group and significant effects of sex and activity were determined by two-way ANOVA; NS, $p > 0.05$, not significant. When a significant interaction was found, values in a row with a different superscript letter are significantly different ($p < 0.01$) as identified by least squares means.

TABLE 3.4 Effect of Diet and Exercise-Training on Immune Cell Phenotypes in Freshly Isolated Rat Splenocytes[1]

| | Sedentary | | Exercise-Trained | | Significance, $p <$ [2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenotype** | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Activity |
| | % of total immune cells in spleen | | | | | |
| CD5$^+$ T cells | 43 ± 1 | 44 ± 2 | 45 ± 2 | 46 ± 1 | NS | NS |
| W3/13$^+$ (pan T cells) | 45 ± 2 | 43 ± 2 | 44 ± 2 | 44 ± 1 | NS | NS |
| CD4$^+$ T helper cells | 28 ± 1 | 27 ± 1 | 29 ± 1 | 28 ± 1 | NS | NS |
| CD8$^+$ T suppressor/cytotoxic cells | 24 ± 1 | 23 ± 1 | 21 ± 1 | 22 ± 1 | NS | NS |
| B cells | 23 ± 1 | 23 ± 1 | 25 ± 1 | 23 ± 1 | NS | NS |
| Macrophages | 4.3 ± 0.5 | 5.4 ± 0.8 | 4.1 ± 0.6 | 4.1 ± 0.6 | NS | NS |
| Natural killer cells | 5.7 ± 0.7 | 4.5 ± 0.7 | 5.4 ± 0.4 | 6.5 ± 0.8 | NS | NS |
| CD4/CD8 | $1.2 \pm 0.04^b$ | $1.2 \pm 0.04^b$ | $1.4 \pm 0.03^a$ | $1.3 \pm 0.03^b$ | NS | 0.01 |

[1] Values are means ± SEM for sedentary and exercise-trained rats fed a low n-3 or high n-3 diet ($n \geq 8$/group). There was no effect of sex on the proportion (% of total) of splenocytes within lymphocyte subsets as determined by three-way ANOVA; therefore, male and female rats were combined within each treatment group.

[2] Significant effect of diet and activity as determined by two-way ANOVA, NS = $p > 0.05$. Values in a row with a different superscript letter are significantly different ($p < 0.05$) as identified by least squares means.

**TABLE 3.5 Effect of Diet and Exercise-Training on Immune Cell Phenotypes in Rat Spleen After Splenocytes Were Cultured for 48 h Without Mitogen**[1]

| | Sedentary | | Exercise-Trained | | Significance, p <[2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenoytpe** | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Activity |
| | % of total immune cells in spleen | | | | | |
| $CD5^+$ T cells | 40 ± 3 | 40 ± 4 | 42 ± 3 | 38 ± 4 | NS | NS |
| $w3/13^+$ (pan T cells) | 38 ± 5 | 40 ± 6 | 37 ± 6 | 40 ± 3 | NS | NS |
| $CD4^+$ T helper cells | 31 ± 1 | 29 ± 3 | 30 ± 1 | 28 ± 3 | NS | NS |
| $CD8^+$ T suppressor/cytotoxic cells | 22 ± 1 | 20 ± 2 | 22 ± 1 | 19 ± 2 | NS | NS |
| B cells | 27 ± 3 | 28 ± 2 | 30 ± 3 | 28 ± 3 | NS | NS |
| Macrophages | 11 ± 3 | 8.8 ± 1.9 | 9.1 ± 2.4 | 9.6 ± 1.2 | NS | NS |
| CD4/CD8 | 1.5 ± 0.1 | 1.4 ± 0.1 | 1.4 ± 0.1 | 1.5 ± 0.1 | NS | NS |

[1] Values are means ± SEM for sedentary and exercise-trained rats fed a low n-3 or high n-3 diet ($n \geq 5$/group). There was no effect of sex on the proportion (% of total) of splenocytes within lymphocyte subsets as determined by three-way ANOVA; therefore, male and female rats were combined within each treatment group.

[2] No significant effects of diet, activity, or their interaction were found (NS = $p > 0.05$, by two-way ANOVA).

**TABLE 3.6 Effect of Diet and Exercise-Training on Immune Cell Phenotypes in Rat Spleen After Splenocytes Were Cultured for 48 h With Concanavalin A**[1]

| | Sedentary | | Exercise-Trained | | Significance, $p <$ [2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenoytpe** | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Activity |
| | % of total immune cells in spleen | | | | | |
| CD5$^+$ T cells | 36 ± 2 | 37 ± 5 | 37 ± 3 | 31 ± 3 | NS | NS |
| w3/13$^+$ (pan T cells) | 33 ± 2 | 40 ± 5 | 34 ± 2 | 35 ± 2 | NS | NS |
| CD4$^+$ T helper cells | 24 ± 4 | 31 ± 4 | 30 ± 6 | 30 ± 3 | NS | NS |
| CD8$^+$ T suppressor/cytotoxic cells | 30 ± 3 | 34 ± 3 | 34 ± 6 | 28 ± 4 | NS | NS |
| B cells | 30 ± 6 | 35 ± 6 | 36 ± 6 | 31 ± 4 | NS | NS |
| Macrophages | 20 ± 6 | 31 ± 11 | 25 ± 6 | 26 ± 5 | NS | NS |
| CD4/CD8 | 0.81 ± 0.09 | 0.89 ± 0.06 | 0.89 ± 0.06 | 1.1 ± 0.04 | NS | NS |

[1] Values are means ± SEM for sedentary and exercise-trained rats fed a low n-3 or high n-3 diet ($n \geq 4$/group). There was no effect of sex on the proportion (% of total) of splenocytes within lymphocyte subsets as determined by three-way ANOVA; therefore, male and female rats were combined within each treatment group.

[2] No significant effects of diet, activity, or their interaction were found (NS = $p > 0.05$, by two-way ANOVA).

**TABLE 3.7 Effect of Cell Culture Condition on Immune Cell Phenotypes in Rat Spleen[1]**

| | | Cell Culture Condition | | | Significance, p <[2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenotype** | $n^3$ | Fresh | 48 h (- Con A) | 48 h (+ Con A) | Culture | Stimulation |
| | | % of total immune cells in spleen | | | | |
| CD5$^+$ T cells | 18 | 45 ± 1 | 43 ± 2 | 35 ± 2 | NS | 0.001 |
| w3/13 (pan T-cells) | 18 | 44 ± 1 | 46 ± 1 | 35 ± 1 | NS | 0.0001 |
| CD4$^+$ T helper cells | 18 | 28 ± 1 | 32 ± 1 | 29 ± 2 | 0.0001 | NS |
| CD8$^+$ T suppressor/cytotoxic cells | 17 | 21 ± 1 | 22 ± 1 | 31 ± 2 | NS | 0.0003 |
| B cells | 18 | 23 ± 1 | 31 ± 1 | 33 ± 3 | 0.0001 | NS |
| Macrophages | 12 | 5.1 ± 0.4 | 8.2 ± 1.0 | 33 ± 3 | 0.01 | 0.0001 |

[1] Values are means ± SEM for freshly isolated splenocytes and splenocytes cultured with (+) or without (-) Concanavalin A (Con A) for 48 h. There was no effect of sex on the proportion (% of total) of splenocytes within lymphocyte subsets as determined by three-way ANOVA; therefore, male and female rats were combined within each treatment group. Neither diet nor exercise-training significantly affected the proportion of splenocytes within lymphocyte subsets; therefore, treatment groups were combined and expressed by cell culture condition.

[2] A paired *t* test was used to compare freshly isolated splenocytes with those cultured without Con A for 48 h (significant effect of culture) and splenocytes cultured without Con A for 48 h with those cultured with Con A for 48 h (significant effect of stimulation). NS, $p > 0.05$, not significant.

[3] *n*, number of samples per cell culture condition for each immune cell phenotype.

**TABLE 3.8 Effect of Diet and Exercise-Training on Immune Cell Phenotypes Expressing the Transferrin Receptor (CD71) in Freshly Isolated Rat Splenocytes**[1]

| | Sedentary | | Exercise-Trained | | Significance, p <[2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenotype** | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Activity |
| | % of total immune cells in spleen | | | | | |
| $CD71^+$ $CD5^+$ T cells | 4.1 ± 0.6 | 4.1 ± 0.3 | 3.7 ± 0.4 | 3.9± 0.5 | NS | NS |
| $CD71^+$ $CD4^+$ T helper cells | 3.5 ± 0.5 | 3.7± 0.4 | 3.2 ± 0.4 | 4.4 ± 0.4 | NS | NS |
| $CD71^+$ $CD8^+$ T suppressor/cytotoxic cells | 3.0 ± 0.4 | 3.4 ± 0.5 | 2.7 ± 0.5 | 3.7 ± 0.6 | NS | NS |
| $CD71^+$ Macrophages | 3.7 ± 0.5 | 3.8 ± 0.4 | 3.6 ± 0.3 | 4.5 ± 0.7 | NS | NS |
| $CD71^+$ CD4/CD8 | 1.1 ± 0.2 | 1.3 ± 0.2 | 1.3 ± 0.2 | 1.3 ± 0.2 | NS | NS |

[1] Values are means ± SEM for sedentary and exercise-trained rats fed a low n-3 or high n-3 diet ($n \geq 6$/group). There was no effect of sex on the proportion (% of total) of splenocytes within lymphocyte subsets as determined by three-way ANOVA; therefore, male and female rats were combined within each treatment group.

[2] No significant effects of diet, activity, or their interaction were found (NS = $p > 0.05$, by two-way ANOVA).

**TABLE 3.9 Effect of Diet and Exercise-Training on Immune Cell Phenotypes Expressing the Transferrin Receptor (CD71) After Splenocytes Were Cultured for 48 h Without Mitogen**[1]

| | Sedentary | | Exercise-Trained | | Significance, p <[2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenoytpe** | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Activity |
| | % of total immune cells in spleen | | | | | |
| $CD71^+$ $CD5^+$ T cells | 7.9 ± 1.3 | 5.9 ± 1.2 | 10 ± 3 | 7.5 ± 1.5 | NS | NS |
| $CD71^+$ $CD4^+$ T helper cells | 14 ± 4 | 11 ± 3 | 9.1 ± 1.7 | 8.6 ± 2.9 | NS | NS |
| $CD71^+$ $CD8^+$ T suppressor/cytotoxic cells | 13 ± 3 | 11 ± 2 | 7.9 ± 0.9 | 9.8 ± 2.8 | NS | NS |
| $CD71^+$ Macrophages | 3.3 ± 0.5 | 4.3± 0.5 | 2.9 ± 0.3 | 3.4 ± 0.2 | NS | NS |
| $CD71^+$ CD4/CD8 | 1.2 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 | 0.93 ± 0.14 | NS | NS |

[1] Values are means ± SEM for sedentary and exercise-trained rats fed a low n-3 or high n-3 diet ($n \geq 4$/group). There was no effect of sex on the proportion (% of total) of splenocytes within lymphocyte subsets as determined by three-way ANOVA; therefore, male and female rats were combined within each treatment group.

[2] No significant effects of diet, activity, or their interaction were found (NS = $p > 0.05$, by two-way ANOVA).

TABLE 3.10 Effect of 48 h Culture (Without Mitogen) on Transferrin Receptor (CD71) Expression in Rat Splenocytes[1]

| Immune Cell Phenotype | $n$[3] | Cell Culture Condition<br>Fresh | <br>48 h (- Con A) | Significance, $p <$[2] |
|---|---|---|---|---|
| | | % of total immune cells in spleen | | |
| $CD71^+$ $CD5^+$ T cells | 15 | 3.8 ± 0.3 | 7.3 ± 0.8 | 0.0001 |
| $CD71^+$ $CD4^+$ T helper cells | 13 | 4.3 ± 0.4 | 11 ± 2 | 0.003 |
| $CD71^+$ $CD8^+$ T suppressor/cytotoxic cells | 14 | 3.8 ± 0.3 | 11 ± 2 | 0.0004 |
| $CD71^+$ Macrophages | 13 | 4.5 ± 0.4 | 3.5 ± 0.2 | 0.04 |

[1] Values are means ± SEM for freshly isolated splenocytes and splenocytes cultured for 48 h without (-) Con A. There was no effect of sex on the proportion (% of total) of splenocytes within lymphocyte subsets as determined by three-way ANOVA; therefore, male and female rats were combined within each treatment group. Neither diet nor exercise-training significantly affected the proportion of splenocytes within lymphocyte subsets; therefore, treatment groups were combined and expressed by cell culture condition.

[2] Significant ($p < 0.05$) effect of culture as determined by paired *t* test comparing freshly isolated splenocytes with those cultured without Con A for 48 h.

[3] *n*, number of samples per cell culture condition for each immune cell phenotype.

**TABLE 3.11 Effect of Diet and Exercise-Training on Fatty Acid Composition of Phosphatidylcholine in Immune Cells of Rats[1]**

| | Sedentary | | Exercise-Trained | | Significance, p <[2] | |
|---|---|---|---|---|---|---|
| **Fatty acid** | **Low n-3** | **High n-3** | **Low n-3** | **High n-3** | **Diet** | **Exercise** |
| | % w/w of total fatty acids | | | | | |
| C18:2 (6) | $12 \pm 0.5$ | $17 \pm 1$ | $12 \pm 0.5$ | $17 \pm 1$ | 0.001 | NS |
| C20:4 (6) | $22 \pm 1^a$ | $14 \pm 1^c$ | $19 \pm 1^b$ | $13 \pm 1^c$ | 0.0001 | 0.01 |
| C20:5 (3) | nd$^c$ | $0.98 \pm 0.12^a$ | $0.42 \pm 0.16^b$ | $0.97 \pm 0.12^a$ | 0.0001 | NS |
| C22:4 (6) | $1.8 \pm 0.1^a$ | $0.67 \pm 0.04^c$ | $1.6 \pm 0.02^b$ | $0.58 \pm 0.02^c$ | 0.0001 | 0.005 |
| C22:5 (6) | $0.30 \pm 0.03$ | nd | $0.30 \pm 0.02$ | nd | 0.0001 | NS |
| C22:5 (3) | $0.42 \pm 0.05$ | $1.8 \pm 0.1$ | $0.54 \pm 0.13$ | $1.8 \pm 0.1$ | 0.0001 | NS |
| C22:6 (3) | $0.71 \pm 0.09$ | $1.7 \pm 0.1$ | $0.80 \pm 0.11$ | $1.6 \pm 0.2$ | 0.0001 | NS |
| SFA | $46 \pm 1$ | $51 \pm 1$ | $47 \pm 1$ | $49 \pm 2$ | 0.005 | NS |
| MUFA | $12 \pm 1$ | $13 \pm 1$ | $14 \pm 1$ | $13 \pm 1$ | NS | NS |
| P/S ratio | $0.92 \pm 0.04$ | $0.73 \pm 0.04$ | $0.86 \pm 0.05$ | $0.78 \pm 0.07$ | 0.05 | NS |
| n-6 PUFA | $41 \pm 1$ | $34 \pm 1$ | $37 \pm 1$ | $33 \pm 2$ | 0.005 | NS |
| n-3 PUFA | $1.3 \pm 0.1$ | $4.4 \pm 0.3$ | $2.0 \pm 0.3$ | $4.5 \pm 0.3$ | 0.0001 | NS |
| n-6/n-3 | $34 \pm 3^a$ | $8.3 \pm 0.9^c$ | $20 \pm 3^b$ | $7.5 \pm 0.6^c$ | 0.0001 | 0.05 |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq$ 5/diet). Abbreviations used: nd, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; n-6 PUFA, sum of n-6 polyunsaturated fatty acids; n-3 PUFA, sum of n-3 polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids

[2] Significant effect of dietary n-3 fatty acids and exercise-training as determined by two-way ANOVA, NS = $p > 0.05$. When an interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

**TABLE 3.12 Effect of Diet and Exercise-Training on Fatty Acid Composition of Phosphatidylethanolamine in Immune Cells of Rats[1]**

| | Sedentary | | Exercise-Trained | | Significance, p <[2] | |
|---|---|---|---|---|---|---|
| Fatty acid | Low n-3 | High n-3 | Low n-3 | High n-3 | Diet | Exercise |
| | % w/w of total fatty acids | | | | | |
| C18:2 (6) | 4.6 ± 0.2 | 6.1 ± 0.2 | 4.4 ± 0.1 | 5.4 ± 0.9 | 0.01 | NS |
| C20:4 (6) | 38 ± 3 | 32 ± 1 | 34 ± 2 | 33 ± 1 | NS | NS |
| C20:5 (3) | 0.22 ± 0.08 | 2.8 ± 0.13 | 0.12 ± 0.08 | 2.9 ± 0.06 | 0.0001 | NS |
| C22:4 (6) | 8.8 ± 0.6$^{a}$ | 2.6 ± 0.2$^{c}$ | 6.7 ± 0.9$^{b}$ | 2.5 ± 0.2$^{c}$ | 0.0001 | NS |
| C22:5 (6) | 1.3 ± 0.1 | 0.10 ± 0.02 | 1.2 ± 0.2 | 0.15 ± 0.01 | 0.0001 | NS |
| C22:5 (3) | 1.5 ± 0.1 | 8.3 ± 0.3 | 1.3 ± 0.2 | 8.8 ± 0.6 | 0.0001 | NS |
| C22:6 (3) | 2.6 ± 0.2$^{c}$ | 6.9 ± 0.4$^{b}$ | 2.5 ± 0.1$^{c}$ | 8.1 ± 0.2$^{a}$ | 0.0001 | NS |
| SFA | 32 ± 2$^{ab}$ | 32 ± 1$^{ab}$ | 37 ± 2$^{a}$ | 30 ± 2$^{b}$ | NS | NS |
| MUFA | 7.8 ± 1.1 | 7.4 ± 0.2 | 10.6 ± 1.8 | 9.4 ± 1.9 | NS | NS |
| P/S ratio | 2.0 ± 0.2$^{a}$ | 1.9 ± 0.1$^{a}$ | 1.3 ± 0.2$^{b}$ | 2.1 ± 0.2$^{a}$ | NS | NS |
| n-6 PUFA | 55 ± 3 | 42 ± 1 | 49 ± 2 | 43 ± 2 | 0.005 | NS |
| n-3 PUFA | 4.8 ± 0.3$^{c}$ | 18 ± 1$^{b}$ | 4.4 ± 0.1$^{c}$ | 20 ± 1$^{a}$ | 0.0001 | NS |
| n-6/n-3 | 12 ± 1 | 2.4 ± 0.1 | 11 ± 0.2 | 2.1 ± 0.03 | 0.0001 | NS |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq$ 5/diet). Abbreviations used are defined in the legend to Table 3.11.

[2] Significant effect of dietary n-3 fatty acids and exercise-training as determined by two-way ANOVA, NS = $p > 0.05$. When an interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

**TABLE 3.13 Effect of Diet and Exercise-Training on Fatty Acid Composition of Phosphatidylinositol in Immune Cells of Rats[1]**

| | Sedentary | | Exercise-Trained | | Significance, p <[2] | |
|---|---|---|---|---|---|---|
| Fatty | Low n-3 | High n-3 | Low n-3 | High n-3 | Diet | Exercise |
| | | | % w/w of total fatty acids | | | |
| C18:2 (6) | 3.4 ± 0.2 | 3.5 ± 0.5 | 3.4 ± 0.1 | 3.5 ± 0.1 | NS | NS |
| C18:3 (3) | 0.10 ± 0.01 | 0.13 ± 0.01 | 0.28 ± 0.06 | 0.25 ± 0.16 | NS | 0.01 |
| C18:4 (3) | nd | nd | 0.20 ± 0.07 | 0.09 ± 0.02 | NS | 0.03 |
| C20:4 (6) | 35 ± 3 | 32 ± 0.5 | 29 ± 2 | 37 ± 2 | NS | NS |
| C20:5 (3) | 0.18 ± 0.05 | 1.0 ± 0.1 | 0.34 ± 0.08 | 1.0 ± 0.2 | 0.0001 | NS |
| C22:4 (6) | $1.8 \pm 0.1^{a}$ | $0.86 \pm 0.01^{c}$ | $1.3 \pm 0.2^{b}$ | $0.86 \pm 0.16^{c}$ | 0.0001 | 0.05 |
| C22:5 (6) | $0.15 \pm 0.01^{a}$ | nd$^{b}$ | $0.12 \pm 0.01^{a}$ | $0.14 \pm 0.05^{a}$ | NS | NS |
| C22:5 (3) | 0.27 ± 0.04 | 1.7 ± 0.1 | 0.16 ± 0.02 | 1.7 ± 0.3 | 0.0001 | NS |
| C22:6 (3) | 0.37 ± 0.06 | 0.67 ± 0.06 | 0.22 ± 0.02 | 0.80 ± 0.07 | 0.0001 | NS |
| SFA | $49 \pm 2^{ab}$ | $47 \pm 1^{bc}$ | $54 \pm 2^{a}$ | $40 \pm 1^{c}$ | 0.004 | NS |
| MUFA | 5.4 ± 0.2 | 7.5 ± 0.7 | 7.5 ± 1.0 | 7.8 ± 0.2 | NS | NS |
| P/S ratio | $0.90 \pm 0.09^{ab}$ | $0.91 \pm 0.03^{ab}$ | $0.63 \pm 0.09^{b}$ | $1.2 \pm 0.01^{a}$ | 0.01 | NS |
| n-6 PUFA | $42 \pm 3^{ab}$ | $38 \pm 1^{ab}$ | $32 \pm 4^{b}$ | $44 \pm 1^{a}$ | NS | NS |
| n-3 PUFA | 0.98 ± 0.07 | 3.7 ± 0.2 | 1.2 ± 0.2 | 3.8 ± 0.2 | 0.0001 | NS |
| n-6/n-3 | 44 ± 5 | 11 ± 0.5 | 29 ± 6 | 11 ± 1 | 0.0003 | NS |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM (*n* ≥ 5/diet). Abbreviations used are as defined in Table 3.11.

[2] Significant effect of dietary n-3 fatty acids and exercise-training as determined by two-way ANOVA, NS = $p > 0.05$. When an interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

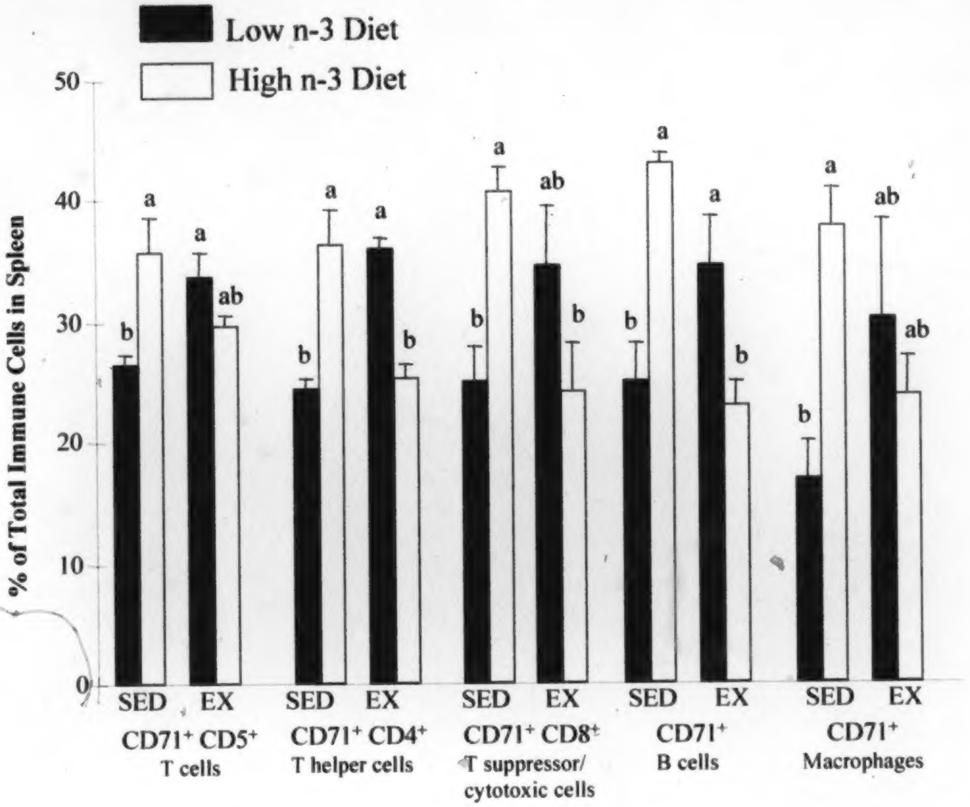

**FIGURE 3.1 Effect of Dietary Long-Chain n-3 Fatty Acids on Transferrin Receptor (CD71) Expression in Splenocytes From Sedentary and Exercise-Trained Rats.** Isolated splenocytes were stimulated for 48 h with Concanavalin A (5 mg/L) and immune phenotypes were identified by indirect immunofluorescence assay and flow cytometry as described in Materials and Methods. Bars represent means ± SEM ($n \geq 5$/group). For each phenotype, the main effects (diet and exercise) were analyzed by two-way ANOVA. For each phenotype, values that do not share a common superscript are significantly different ($p < 0.05$) as identified by least squares means.

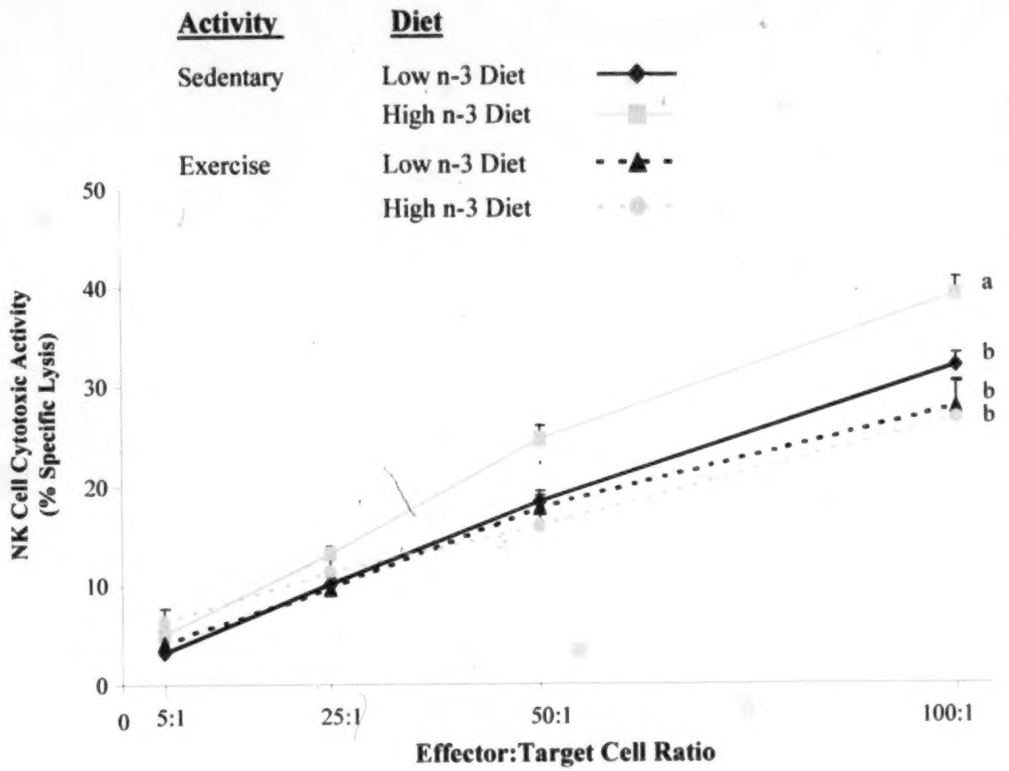

**FIGURE 3.2 Effect of Dietary Long-Chain n-3 Fatty Acids and Exercise-Training on Splenocyte Natural Killer Cell Cytotoxicity.** Natural killer (NK) cell cytotoxic activity is expressed as % specific lysis = 100 X (mean experimental $^{51}$Cr release from labeled YAC-1 cells - mean spontaneous $^{51}$Cr release)/(mean maximum $^{51}$Cr release - mean spontaneous $^{51}$Cr release). Points represent means ± SEM (Sedentary, $n \geq 11$/diet; Exercise-trained, $n = 5$/diet). Lines that do not share a common letter are significantly different ($p < 0.05$) as determined by a split-plot (repeated measures) ANOVA.

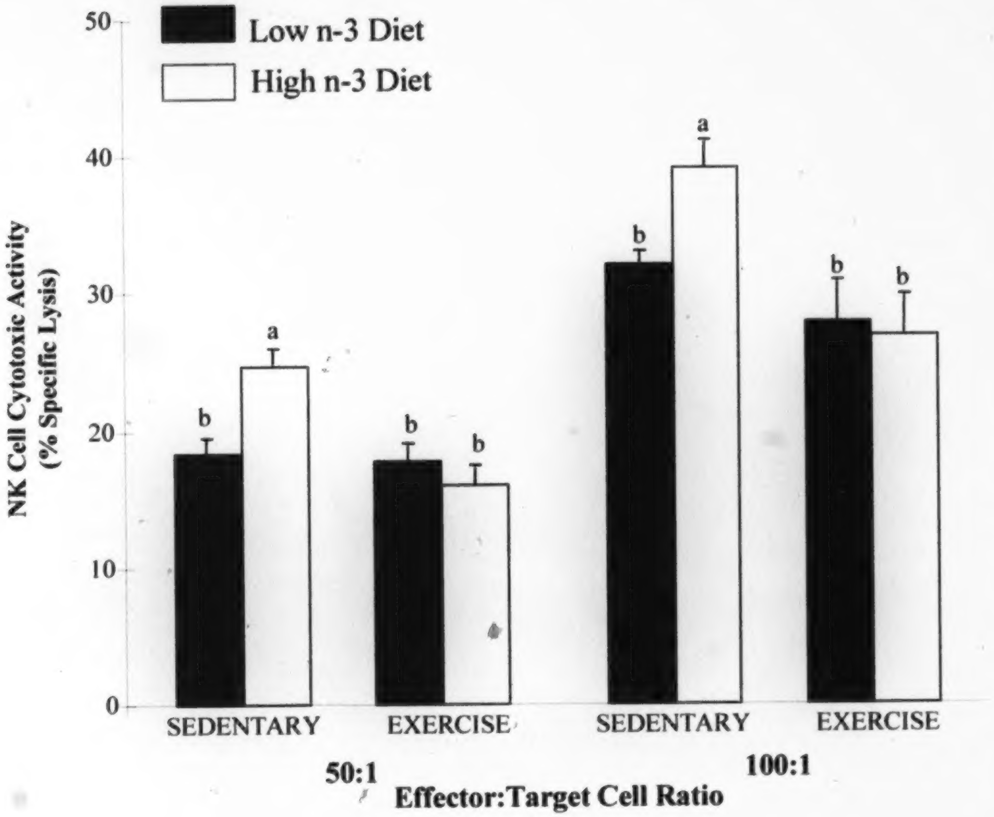

**FIGURE 3.3 Effect of Dietary Long-Chain n-3 Fatty Acids and Exercise-Training on Splenocyte Natural Killer Cell Cytotoxicity at the 50:1 and 100:1 Effector:Target Cell Ratios.** Natural killer (NK) cell cytotoxic activity against YAC-1 cells at the 50:1 and 100:1 effector:target cell ratios is expressed as % specific lysis = 100 X (mean experimental $^{51}$Cr release from labeled YAC-1 cells - mean spontaneous $^{51}$Cr release)/(mean maximum $^{51}$Cr release - mean spontaneous $^{51}$Cr release). Values are means ± SEM (Sedentary, *n* = 12/diet; Exercise-trained, *n* = 5/diet). For each effector:target cell ratio, the main effects (diet and exercise) were analyzed by two-way ANOVA. For each effector:target cell ratio, values that do not share a common superscript are significantly different ($p < 0.001$) as identified by least squares means.

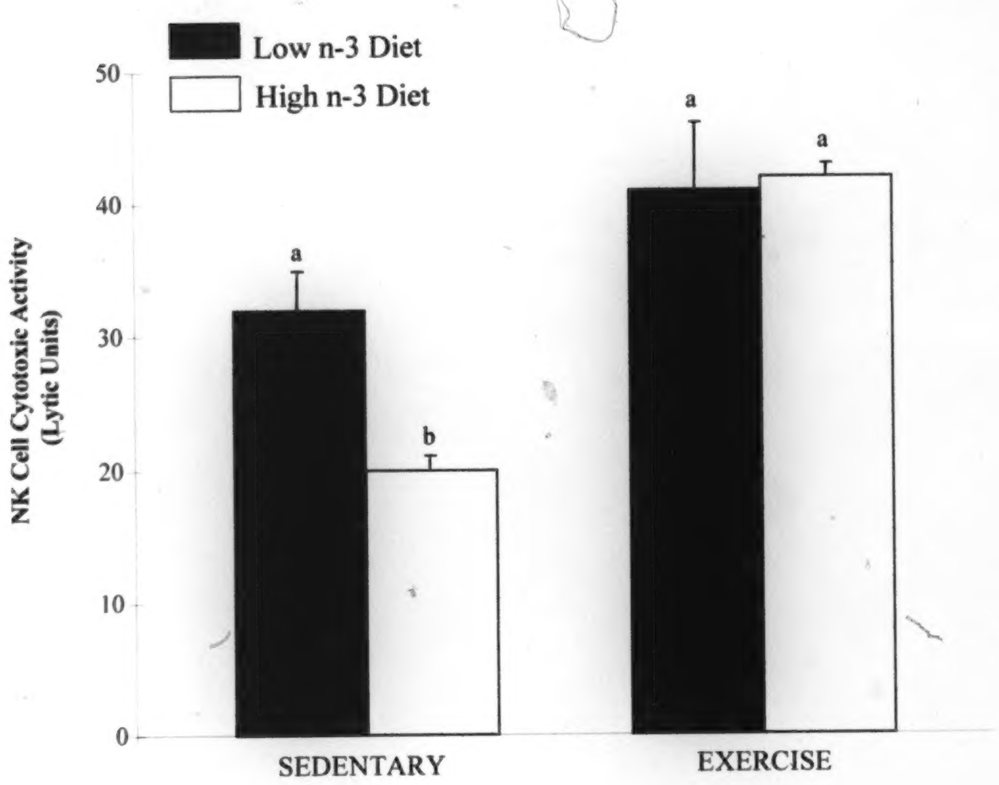

**FIGURE 3.4 Effect of Dietary Long-Chain n-3 Fatty Acids on Splenocyte Natural Killer Cell Lytic Activity of Sedentary and Exercise-Trained Rats.** Lytic units are defined as the number (x $10^{-3}$) of natural killer (NK) cells required to cause 20% lysis of target cells. Values are means ± SEM (Sedentary, *n* = 12/diet; Exercise-trained, *n* = 5/diet). The main effects (diet and exercise) were analyzed by two-way ANOVA. Values that do not share a common superscript are significantly different ($p < 0.05$) as identified by least squares means.

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# 4. DIETARY LONG-CHAIN n-3 FATTY ACIDS ALTER PHOSPHOLIPID COMPOSITION AND FUNCTION OF PRE-ACTIVATED AND ACTIVATED IMMUNE CELLS[1,2]

## 4.1. INTRODUCTION

Diets rich in the fish oil-derived long-chain n-3 polyunsaturated fatty acids, eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids, can affect both innate (natural) and acquired (specific) cell-mediated immune responses (Calder 1998). For example, animals fed high levels of EPA and DHA have suppressed natural killer (NK) cell cytotoxic activity (Jeffery et al. 1996, Sanderson et al. 1995, Yaqoob et al. 1994a, Meydani et al. 1988), reduced lymphocyte mitogenic response (Jeffery et al 1996, Sanderson et al 1995, Yaqoob et al. 1994b) and decreased cytotoxic T cell activity (Fritsche and Johnston 1990, Olson et al. 1987). Overall, studies have concluded that consumption of large amounts of long-chain n-3 fatty acids is immunosuppressive. However, the physiological relevance of these effects is not clear, since many previous studies have used single dietary oils or mixtures of two oils which often resulted in levels of n-3 fatty acids far above those consumed by most humans as well as very low intakes of the essential n-6 fatty acid, linoleic acid (C18:2n-6).

The mechanism for the effect of n-3 fatty acids on the immune system may involve modulation of membrane-mediated functions through changes in membrane lipid composition (Calder 1998, Peck 1994). It is well established that changes in dietary fat composition can induce significant alterations in the composition and function of membranes in a variety of human and animal tissues (Field et al. 1988, Clandinin et al. 1985). In lymphocytes, membrane-associated events play a pivotal role in signal transduction (Jolly et al. 1997, Hosack-Fowler et al. 1993), the expression of surface markers (Jenski et al. 1995, Yaqoob et al 1994b) and cellular activation (Calder et al. 1994, Hadden 1988), all of which are important in immune cell function during healthy

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[2] This work was presented in part at the AOCS Annual Meeting and Expo, May 1997, Seattle, WA (Robinson, L. E. and Field, C. J. Improving immune anticancer defenses by feeding long chain n-3 fatty acids).

states and disease conditions such as cancer. Also, many functions of the immune system depend on interactions between effector and target cell membranes. Thus, changing the membrane composition of such cells, through modulating dietary lipids, may influence immune responses. More specifically, there is evidence to support the claim that n-3 and n-6 polyunsaturated fatty acid composition of lymphocyte membranes influences immune function (Jenski et al 1995, Yaqoob et al 1994b). Again, these reports dealt with only very large changes in the n-6/n-3 content of immune cells. The effect of smaller changes in membrane lipid on immune function as a result of including a more physiological level of n-3 fatty acids in the diet is not known. Furthermore, despite the fact that most functional assays used to determine the role of dietary fat in modulating immune function are performed 48-72 h after removal of cells from the diet-fed animal, few studies have examined immune cell membrane changes that occur or are maintained during culture or activation.

The objectives of this study were to determine the effects of a physiological (with respect to human diets) change in long-chain n-3 fatty acid intake on membrane lipid composition of immune cells in the pre-activated and activated states and membrane-mediated immune defense involved in immune activation.

## 4.2. MATERIALS AND METHODS

**Animals and diets.** Experiments were reviewed and approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Eight male (190 ± 13 g) and 8 female (142 ± 5 g) adult Fischer 344 rats (Charles River Laboratories, St. Constant, QB, Canada) were housed in individual plastic shoebox cages in a temperature controlled room (23 °C) maintained on a 12 h light/dark cycle. Body weight and food intake were recorded every third day throughout the study. Rats were randomly assigned to be fed one of two nutritionally complete, semi-purified diets (Teklad Test Diets, Madison, WI) containing (per kg) 270 g high protein casein, 408 g carbohydrate and 200 g fat. The complete nutrient composition of the diets was described in Chapter 3. Briefly, the two experimental diets differed only in the composition of fat, providing two different levels of long chain n-3 fatty acids from fish oil (P-28 Nisshin lot # 28020, Nisshin Flour Milling Co., Ltd., Tokyo, Japan): low (0 g/kg) or high (51 g/kg of total fat). The fatty acid composition of the diets is presented in **Table 4.1**. All animals were given free access to food and water for 4 wk. All assays were performed on individual rats (*n* = 8/treatment group unless otherwise indicated).

**Splenocyte isolation and activation.** Following $CO_2$ asphyxiation and cervical dislocation, the spleen from each animal was removed and weighed. Splenocytes were isolated aseptically in Krebs-Ringer HEPES buffer (KRH, pH 7.4) supplemented with bovine serum albumin (BSA, 5 g/L; Sigma Chemical, St. Louis, MO) as described in Chapter 3. Isolated splenocytes (2.5 x $10^9$ cells/L) in complete culture media (CCM; defined in Chapter 3) were incubated in 24-well sterile plates (Costar®, Corning Glass Works, Corning, NY) for 48 h in a humidified atmosphere at 37 °C in the presence of 5% $CO_2$. The cell culture media contained either no mitogen (unstimulated cells) or was supplemented with Concanavalin A (Con A, 5 mg/L; ICN, Montréal, QB, Canada) or Phorbol Myristate Acetate (PMA, 30 µg/L; ICN) plus Ionomycin (0.75 µmol/L; Sigma Chemical). After 48 h, splenocytes were centrifuged at 228 g (Beckman J2-HC, Beckman Instruments, Palo Alto, CA) for 10 min at 4 °C. Splenocyte culture supernatants were collected and stored at -70 °C for subsequent nitric oxide and cytokine analyses. Pelleted splenocytes (unstimulated and stimulated) were washed twice in KRH (pH 7.4) supplemented with BSA (5 g/L) and frozen at -70 °C for subsequent lipid analysis.

**Fatty acid analysis.** Lipids were extracted from splenocytes (freshly isolated, post-culture unstimulated and mitogen-stimulated) by a modified Folch (Folch et al. 1957) procedure (Field et al 1988) as described in Chapter 3. Individual phospholipids were separated on Whatman HPK thin layer chromatography plates (10 x 10 cm) and compared with appropriate standards as described in Chapter 3. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) fatty acid methyl esters were prepared using 14% (w/v) $BF_3$/methanol reagent (Morrison and Smith 1964) and separated by automated gas liquid chromatography as described in Chapter 3.

**Indirect immunofluorescence (phenotype) assay.** Immune cell subsets from splenocytes were identified by indirect immunofluorescence assay as described in Chapter 3. The monoclonal antibodies used were the same as those defined in Chapter 3 and are defined in **Table 4.2.**

**Splenocyte mitogenic response (proliferation) assay.** Splenocytes (1.25 x $10^9$ cells/L) were cultured in 96-well microtiter plates (Corning) with or without either Con A (5 mg/L) or PMA (30 µg/L) plus Ionomycin (0.75 µmol/L) for 66 h as previously described (Shewchuk et al. 1996). Twelve hours prior to harvesting the cells, each well was pulsed with 18.5 kBq of [methyl-$^3$H]-thymidine (Amersham, Oakville, ON, Canada). Cells were incubated for an additional 12 h before being harvested onto glass fibre filters (Skatron, Suffolk, UK) using a multiwell harvester (Skatron, Lier, Norway). The dried filters were dissolved in 4 mL Ecolite® scintillation cocktail (ICN) and radioactivities were measured using a betacounter (Beckman, LS 5801 Beckman Instruments, Inc., Mississauga, ON, Canada). All assays were performed in triplicate and stimulation indices were calculated for each condition as [amount of [$^3$H]-thymidine (kBq/min) incorporated by stimulated cells - amount of [$^3$H]-thymidine (kBq/min) incorporated by unstimulated cells]/amount of [$^3$H]-thymidine (kBq/min) incorporated by unstimulated cells.

**Interleukin-2 (IL-2) production**. IL-2 concentration in culture supernatants collected from unstimulated and mitogen-stimulated splenocytes was determined by a colorimetric enzyme-linked immunosorbent assay (ELISA). All antibodies and standards were purchased from PharMingen (Mississauga, ON, Canada). Supernatants were prepared and frozen at -70 °C as described above. Purified rabbit anti-rat IL-2 capture antibody was diluted to 2 µg/mL in binding solution (see Appendix B for composition of all solutions in this protocol) and 50 µL was added to each well of high binding flat-bottom microtiter plates (Immulon®, Dynex Technologies, Inc., Chantilly, VA). Plates were sealed, incubated overnight at 4 °C, and washed 4 times with wash buffer. Each wash involved adding 200 µL of wash buffer, gently shaking for 1 min, and inverting and blotting the plate on an absorbent surface. Non-specific binding was blocked by adding

200 μL of blocking buffer/well, sealing the plates and incubating at room temperature for 30 min. Plates were then washed 3 times as described above. Recombinant standards were diluted in blocking buffer and 100 μL was added to each well (15-2000 pg/mL). Stimulated samples were added (50 μL/well) and diluted with 50 μL of blocking buffer, whereas unstimulated samples were added (100 μL/well) so that all wells contained equal volumes. Differences in the amount of supernatant added were accounted for in the final calculations. Standards and samples were added to wells in triplicate. Plates were then sealed, incubated for 3 h at room temperature and washed 4 times. Biotinylated mouse anti-rat IL-2 detecting antibody was diluted to 2 μg/mL in blocking buffer and 100 μL was added to each well. Plates were sealed, incubated at room temperature for 1 h and washed 6 times. Horseradish peroxidase-streptavidin was diluted to 0.5 μL/mL in blocking buffer and 100 μL/well was added, plates were sealed, incubated at room temperature for 30 min, and washed 8 times. ABTS Substrate Solution was thawed 20 min prior to use and 10 μL of 30% $H_2O_2$/11 mL of substrate was added and vortexed. Then, 100 μL of this solution was added to each well and plates were incubated at room temperature for 30 min. The optical density was read at 405 nm on a microplate reader (Model EL-309; Bio-Tek Instruments Inc., Burlington, VT). The concentration of IL-2 in splenocyte culture supernatants (unstimulated and stimulated) was quantified by comparison to the standard curve generated with recombinant IL-2.

**NK cell cytotoxicity assay.** A 4 h sodium chromate ($^{51}$Cr, Amersham) release assay was performed using NK cell sensitive YAC-1 murine lymphoma cells (American Type Culture Collection, Rockville, MD) as targets and freshly isolated splenocytes as effector cells as described in Chapter 3.

**Nitric oxide production.** Nitric oxide (NO) production in culture supernatants collected from unstimulated and mitogen-stimulated splenocytes was determined by analyzing nitrite ($NO_2^-$, a product of the L-arginine-dependent NO pathway) concentration using a colorimetric assay based on the Griess reaction (Green et al. 1982). Splenocyte supernatants were prepared and frozen at –70 °C as described above. All Griess reagent chemicals were obtained from Sigma Chemical. Splenocyte culture supernatant (100 μL) or sodium nitrite standard (100 µL) was combined with 100 µL of Griess reagent (1 volume of 0.5% sulfanilamide in 6% phosphoric acid plus 1 volume of 0.05% naphthylethylene-diamine dihydrochloride in distilled water) in a 96-well non-sterile, flat-bottom microtiter plate (Fisher Scientific, Edmonton, AB, Canada) and incubated for 10 min at room temperature. All samples and standards were added in triplicate. The absorbance was measured at 540 nm in an automatic microplate reader (Model EL309; Bio-Tek Instruments, Burlington, VT) and $NO_2^-$ concentrations were determined with reference to a standard curve generated with sodium nitrite (10 to 100 nmol/mL) in complete culture media.

**Statistical analysis.** Results are presented as means ± SEM. All statistical analyses were conducted using the SAS statistical package (Version 6.11, SAS Institute, Cary, NC). The effect of sex was determined by a two-way ANOVA. If no effect of sex was found, the main effect of diet was analyzed by a Student's *t*-test. Food intake, body weight, and NK cell cytotoxicity were compared between groups by a split-plot (repeated measures) ANOVA (Steele and Torrie 1980). For phospholipid data, a two-way ANOVA was used to compare freshly isolated splenocytes to those cultured without Con A for 48 h (significant effect of culture, $p < 0.05$). Similarly, a two-way ANOVA was used to compare splenocytes cultured without Con A for 48 h to those cultured with Con A for 48 h (significant effect of stimulation, $p < 0.05$). Significant differences ($p < 0.05$) between treatments were identified by a Duncan's multiple range test (Steele and Torrie 1980).

## 4.3. RESULTS

**Animal characteristics.** In both diet groups, male rats had a significantly higher final body weight, weight increase and spleen weight compared with female rats (**Table 4.3**). Within each sex, diet did not significantly affect final body weight, weight increase, or spleen weight (Table 4.3). There was no effect of sex or diet on relative food intake (g/kg body weight/d) or the number of spleen cells isolated per gram of spleen (Table 4.3).

**Fatty acid composition of freshly isolated splenocytes.** Sex did not significantly affect the fatty acid composition of splenocytes; therefore, male and female rats were combined within each diet group (**Tables 4.4-4.6**). Splenocytes from rats fed the high n-3 diet had a significantly higher total n-3 content and a significantly lower n-6/n-3 ratio in PC (Table 4.4), PE (Table 4.5) and PI (Table 4.6) compared with rats fed the low n-3 diet. Furthermore, splenocytes from rats fed the high n-3 diet had a significantly lower total n-6 content in PC and PE, but not PI (Tables 4.4-4.6). Specifically, freshly isolated splenocyte PC, PE and PI from rats fed the high n-3 diet had an increased % of C20:5(3), C22:5(3) and C22:6(3) and a decreased % of C22:4(6) and C22:5(6) (Tables 4.4-4.6). In splenocyte PC and PE from rats fed the high n-3 diet, the % of C18:2(6) and C20:4(6) were significantly increased and decreased, respectively (Tables 4.4-4.5), while diet did not affect the % of these fatty acids in PI (Table 4.6). The total saturated fatty acid content and polyunsaturated/saturated fatty acid (P/S) ratio in freshly isolated splenocyte PE and PI were not affected by diet (Tables 4.5-4.6). However, in PC, the total saturated fat content and P/S ratio were significantly increased and decreased, respectively, when rats were fed the high n-3 diet compared with the low n-3 diet (Table 4.4). The total monounsaturated fatty acid content in PI was significantly higher in splenocytes from rats fed the high n-3 diet (Table 4.6), but was not affected by diet in PC and PE (Tables 4.4-4.5).

### Fatty Acid Composition of Splenocytes 48 h Post-Culture (Without Con A)

***Diet effects.*** After 48 h in culture without Con A, splenocytes from rats fed the high n-3 diet had a significantly higher total n-3 content and a significantly lower n-6/n-3 ratio in PC (Table 4.4), PE (Table 4.5) and PI (Table 4.6) compared with rats fed the low n-3 diet. Furthermore, splenocytes from rats fed the high n-3 diet had a significantly lower total n-6 content in PC and PE, but not PI (Tables 4.4-4.6). Specifically, after culture without Con A, splenocyte PC, PE and PI from rats fed the high n-3 diet had a significantly higher % of C22:5(3) and C22:6(3), while dietary long-chain n-3 fatty acids increased the % of C20:5(3) in PC and PE, but not PI (Tables 4.4-4.6). There was no diet effect on the % of C18:2(6) or C20:4(6) in PE or PI in splenocytes cultured without

Con A (Tables 4.5-4.6). However, the % of C18:2(6) and C20:4(6) in PC were significantly increased and decreased, respectively in high n-3-fed rats compared with low n-3-fed rats (Table 4.4). There was no change in the % of C22:4(6) in PC (Table 4.4) or PI (Table 4.6), but the % of C22:4(6) was significantly decreased in PE in rats fed the high n-3 diet (Table 4.5). Dietary long-chain n-3 fatty acids decreased the % of C22:5(6) in PE (Table 4.5) and PI (Table 4.6), but not PC (Table 4.4). The total saturated and monounsaturated fatty acid content and P/S ratio in PC, PE and PI were not affected by diet in splenocytes cultured without Con A (Tables 4.4-4.6).

***Culture effects.*** After 48 h in culture without Con A, the % of C18:2(6) and the total n-6 fatty acid content in PC, PE and PI were significantly decreased for both low and high n-3-fed rats (Tables 4.4-4.6). In PE and PI, the % of C20:4(6) was significantly decreased post-culture for both low and high n-3-fed rats (Tables 4.4-4.6), while in PC, the % of C20:4(6) was significantly decreased only in the low n-3 diet group (Table 4.4). Overall, the ratio of n-6/n-3 fatty acids in PC, PE and PI was significantly decreased after culture for rats fed the low n-3, but not the high n-3, diet (Tables 4.4-4.6). For both diet groups, there was no effect of culture on the total n-3 fatty acid content in PC (Table 4.4). However, in PE and PI the % of C22:5(3) and the total n-3 content were significantly decreased post-culture for rats fed the high n-3, but not the low n-3, diet (Tables 4.5-4.6). The total saturated fat content and P/S ratio in PC and PE were significantly increased and decreased, respectively following culture without Con A (Tables 4.4-4.5). The total saturated fat content in PI was not affected by culture, although the P/S ratio was significantly decreased post-culture for both diet groups (Table 4.6). In PC, PE and PI, the total monounsaturated fat content was significantly increased following culture without Con A for both diet groups (Tables 4.4-4.6).

### Fatty Acid Composition of Splenocytes 48 h Post-Culture (With Con A)

***Diet effects.*** After 48 h stimulation with Con A, splenocytes from rats fed the high n-3 diet had a significantly higher % of C18:2(6), C20:5(3), C22:5(3) and C22:6(3) in PC (Table 4.4) and PE (Table 4.5) compared with rats fed the low n-3 diet. In PI, the % of

C22:5(3) and C22:6(3) were significantly increased in rats fed the high n-3 diet (Table 4.6). In addition, the total n-3 content was significantly higher and the n-6/n-3 ratio was significantly lower in Con A-stimulated splenocyte PC, PE and PI from high n-3-fed rats (Tables 4.4-4.6). In PC, the % of C20:4(6) and C22:5(6) were significantly decreased in high n-3-fed rats, but there was no effect of diet on the % of C22:4(6) (Table 4.4). In PE, there was no diet effect on the % of C20:4(6), but the % of C22:4(6) and C22:5(6) were significantly reduced in high n-3-fed rats (Table 4.5). Diet did not significantly affect the % of C20:4(6), C22:4(6) or C22:5(6) in Con A-stimulated splenocyte PI (Table 4.6). The total n-6, saturated and monounsaturated fatty acid contents and P/S ratio in PC, PE and PI were not significantly affected by diet after splenocytes were stimulated with Con A (Tables 4.4-4.6).

***Stimulation effects.*** After Con A stimulation, the % of C20:4(6) and C20:5(3) and the total n-6, total n-3, and saturated fatty acid contents in PC were significantly decreased for both low and high n-3-fed rats, while the total monounsaturated fatty acid content in PC was significantly increased (Table 4.4). In PE, splenocytes from high n-3-fed, but not low n-3-fed, rats had a significantly higher % of C22:5(3), C22:6(3) and total n-3 content after Con A stimulation (Table 4.5). Also, the % of C18:2(6) and C22:5(6) in PE were significantly increased and decreased, respectively after Con A stimulation of splenocytes from rats fed the low n-3 and high n-3 diets (Table 4.5). In both diet groups, stimulation with Con A significantly decreased the total saturated fat content of PE (Table 4.5). For both low and high n-3-fed rats, the % of C20:5(3) in PI was significantly lower, while the % of C22:5(3), C22:6(3), and C22:5(6) in PI were significantly increased after Con A stimulation (Table 4.6). In both diet groups, the total saturated fat content of PI was not affected by Con A stimulation (Table 4.6). Similarly, in both diet groups, the total monounsaturated fat content of PE and PI was not affected by Con A stimulation (Tables 4.5-5.6).

**Immune cell phenotypes in spleen.** Neither sex nor diet significantly affected the relative proportion (% of total) of $CD8^+$ T suppressor/cytotoxic cells (22 ± 1 %), B cells (34 ± 1 %), macrophages (3.4 ± 0.5%), NK cells (5.0 ± 0.5 %), or the CD4/CD8 ratio (1.2

± 0.1) ($n \geq 13$) (**Table 4.7**). The only sex difference found was that, in the low n-3 diet group, male rats had a significantly lower relative proportion of CD5$^+$ T cells (43 ± 2 % vs 49 ± 2 % for male and female rats, respectively; $n$ = 4/sex) and CD4$^+$ T helper cells (24 ± 1 % vs 28 ± 1 % for male and female rats, respectively; $n$ = 4/sex) (Table 4.7). In the high n-3 diet group, sex did not affect the relative proportion of either CD5$^+$ T cells (46 ± 1 %; $n$ = 8) or CD4$^+$ T cells (27 ± 1 %; $n$ = 8) (Table 4.7). There was no effect of diet on the relative proportion of either CD5$^+$ or CD4$^+$ T cells (Table 4.7).

**Splenocyte mitogenic response.** Sex of the rat did not significantly affect splenocyte proliferative response to Con A at 66 h; therefore, male and female rats were combined within each diet group. Splenocytes from rats fed the high n-3 diet had a significantly lower response (stimulation index) to Con A at 66 h (**Figure 4.1**). There was a significant influence of sex on splenocyte proliferative response to PMA plus Ionomycin at 66 h; splenocytes from female rats fed the low n-3 diet had a significantly lower stimulation index compared with cells from male rats fed the same diet (**Figure 4.2**). Sex of the rat did not significantly affect splenocyte proliferative response to PMA plus Ionomycin in the high n-3 diet group (Figure 4.2). Diet did not significantly affect splenocyte proliferative response to PMA plus Ionomycin at 66 h (Figure 4.2).

**IL-2 production.** For all groups, splenocytes cultured for 48 h without mitogen produced undetectable levels of IL-2 in the culture supernatants (results not shown). In both diet groups, splenocytes from male rats produced lower ($p < 0.0001$) levels of IL-2 after Con A stimulation relative to cells from female rats (**Figure 4.3**). Diet did not affect IL-2 production from Con A-stimulated splenocytes (Figure 4.3). In the low n-3 diet group, PMA plus Ionomycin-stimulated splenocytes from male rats produced lower ($p < 0.002$) levels of IL-2 relative to cells from female rats (Figure 4.3). However, sex did not significantly affect splenocyte IL-2 production after PMA plus Ionomycin stimulation in the high n-3 diet group (Figure 4.3). For female rats, those fed the high n-3 diet had lower ($p < 0.03$) splenocyte IL-2 production after PMA plus Ionomycin stimulation compared with those fed the low n-3 diet (Figure 4.3).

**NK cell cytotoxicity.** Sex of the rat did not significantly affect NK cell cytotoxicity; therefore, male and female rats were combined within each diet group. At all effector:target cell ratios, cells from rats fed the high n-3 diet had a higher ($p < 0.0001$) % specific lysis of target YAC-1 cells relative to those fed the low n-3 diet (**Figure 4.4**). Lytic units (the number of effector cells x $10^{-3}$ required to cause 20 % lysis of YAC-1 cells) were lower ($p < 0.04$) for rats fed the high n-3 diet (20 ± 1, *n* = 5) compared with those fed the low n-3 diet (31 ± 4, *n* = 6) (**Figure 4.5**).

**NO production.** Sex of the rat did not significantly affect splenocyte nitrite ($NO_2^-$) production; therefore, male and female rats were combined within each diet group. For both diet groups $NO_2^-$ production was higher ($p < 0.001$) from splenocytes stimulated with PMA plus Ionomycin compared with unstimulated cells (**Figure 4.6**, statistics not shown). Rats fed the high n-3 diet had increased ($p < 0.05$) splenocyte $NO_2^-$ production by both unstimulated and PMA plus Ionomycin-stimulated splenocytes (Figure 4.6). $NO_2^-$ production from Con A-stimulated splenocytes was not significantly affected by diet (7.5 ± 0.3 nmol/mL/$10^6$ cells, *n* = 8).

## 4.4. DISCUSSION

The n-6 and n-3 polyunsaturated fatty acids have been widely studied for their potential to modulate immune function (Calder 1998). However, by using single dietary oils or mixtures of two oils, many previous studies have investigated the effects of large amounts of n-3 or n-6 polyunsaturated fatty acids, levels which could not be readily achieved in the human diet. In the present study, we determined the effects of a physiological (with respect to human diets) change in long-chain n-3 fatty acid intake on immune cell membrane composition and membrane-mediated immune defense. Healthy rats were fed high fat (200 g/kg) diets containing different amounts of n-3 polyunsaturated fatty acids (0 versus 5g/100g). The diets were formulated to investigate the effect of replacing LA (C18:2n-6) with long-chain n-3 fatty acids (primarily EPA and DHA) or, in effect, altering the dietary n-6/n-3 fatty acid ratio.

Our study confirms previous findings that dietary fat significantly alters the fatty acid composition of immune cell phospholipids (Field et al. 2000, Peterson et al. 1998, Hosack-Fowler et al 1993). In particular, feeding dietary fish oil substantially altered the levels of n-3 and n-6 fatty acids in the major membrane phospholipids PC, PE and PI. As expected, immune cells from rats fed fish oil-derived long-chain n-3 fatty acids had a significantly higher total n-3 content and lower n-6/n-3 fatty acid ratio in PC, PE and PI (Tables 4.4-4.6). Furthermore, many of the diet-induced changes in the fatty acid composition of immune cell phospholipids were maintained during culture and activation with Con A, a polyclonal T cell mitogen. For example, splenocytes isolated from rats fed the high n-3 diet had a significantly higher total n-3 content and lower n-6/n-3 ratio in PC, PE and PI even after cells were cultured for 48 h in the absence or presence of Con A (Tables 4.4-4.6). Our findings are important since investigation of the role of dietary fat in modulating immune function typically involves *in vitro* mitogen stimulation for 48-72 h after removal of cells from animals fed different diets. Thus, we have demonstrated that our *in vitro* system is a valid means of studying the role of dietary long-chain n-3 fatty acids in modulating membrane-mediated immune functions since diet-induced changes in membrane phospholipid composition are maintained in mitogen-activated splenocytes.

In addition to any diet-induced membrane changes, cell activation results in both *de novo* synthesis and an increased turnover of membrane phospholipids (Goppelt-Strübe and Resch 1987) with changes in fatty acid composition of phospholipids observed within 4 h of mitogen contact (Goppelt-Strübe and Resch 1987). We further investigated culture- and mitogen-induced alterations in immune cell membrane composition which occur over an extended period since *in vitro* immune assays are often carried out over 48-72 h. In general, the polyunsaturated fatty acids were those most affected during 48 h culture and activation. For example, cell culture induced a significant decrease in C18:2(6) and C20:4(6) as well as total n-6 fatty acids in splenocyte PC, PE and PI (Tables 4.4-4.6) and Con A stimulation further decreased n-6 fatty acids in PC (Table 4.4). This culture-induced reduction in the essential fatty acid, LA, has important implications for diet and immune function studies as it further emphasizes the need for supplying immune cells with sufficient essential fatty acids prior to *in vitro* cell culture. Other influences of culture on immune cell phospholipids were related to dietary fat composition. For example, culturing splenocytes for 48 h induced a significant decrease in the n-6/n-3 fatty acid ratio in PC, PE and PI, but only in cells from rats fed the low n-3 diet (Tables 4.4-4.6). It is possible that the observed higher rate of cell proliferation ([$^3$H]-thymidine uptake, Figure 4.1) in splenocytes from rats fed the low n-3 diet may have altered membrane n-6 fatty acids since cells require n-6 fatty acids for synthesis of new membranes during proliferation. Con A stimulation increased n-3 fatty acids in PE, but only in splenocytes from high n-3-fed, not low n-3-fed, rats. Since different initial membrane compositions of immune cells may respond differently to culture and activation, our findings raise an important concern regarding the starting composition of cells used to study immune functions *in vitro*. It is possible that different starting amounts of n-3 and n-6 fatty acids (reflective of dietary intake) in immune cell membranes prior to culture and activation could account for discrepancies between our study and a previous study which found that the fatty acid composition of rat lymphocytes was not altered 48 h post-culture (without mitogen) (Calder et al 1994). In the present study, even our "low n-3" (control) diet had a higher n-3 fatty acid content [C18:3(3)] compared with many previous studies using single dietary oils (e.g. corn oil) with low n-3 fatty acids (Fritsche et al. 1999, Yaqoob et al 1994b, Meydani et al 1988).

It is well established that dietary fat manipulation can affect membrane-associated events which play an important role in signal transduction (Jolly et al 1997, Hosack-Fowler et al 1993), expression of cell surface markers (Jenski et al 1995) and cell activation (Calder et al 1994). Since we showed that diet-induced changes in immune cell phospholipid composition can be maintained in an activated state, this suggests a mechanism by which dietary fatty acids affect immune cell activation and function. In the present study, NK cell cytotoxicity was significantly enhanced in rats fed the high n-3 diet (Figure 4.4). Since the percentage of NK ($3.2.3^+$) cells present in the splenocyte population was not affected by diet (Table 4.7), the increased cytotoxicity in high n-3-fed rats may be due to a functional enhancement at the cellular level (lower lytic units, Figure 4.5). Early NK cell activation events are associated with phosphoinositide breakdown and activation of protein kinase C (Chow et al. 1988, Steele and Brahmi 1988). There is also evidence that C20:4(6) plays a role in NKR-P1A-induced NK cell granule release and cell-mediated cytotoxicity (Cifone et al. 1997). Since dietary long-chain n-3 fatty acids substantially altered membrane phospholipid composition, including C20:4(6), signaling events involved in NK cell function may have been affected in rats fed the high n-3 diet, however this requires further study. Upregulation of cytotoxicity may also be associated with immune cell production of cytokines and nitric oxide (Xiao et al. 1995, Whiteside and Herberman 1995, Ortaldo et al. 1984). In rats fed the high n-3 diet, nitric oxide production by unstimulated and PMA plus Ionomycin-stimulated splenocytes was increased (Figure 4.6). Consistent with our results, increased nitric oxide production, in reponse to lipopolysaccharide stimulation, has also been found in mice fed a 20% w/w fish oil diet (Yaqoob and Calder 1995). Although nitric oxide has been proposed as a putative mediator of NK cell cytotoxicity (Cifone et al. 1995), further work using our model is required.

Many previous studies have reported that long-chain n-3 fatty acids (primarily EPA and DHA) decrease proliferative responses to mitogens (Khalfoun et al. 1998, Calder 1996, Meydani et al. 1991) and IL-2 production (Jolly et al. 1998, Meydani et al 1991). However, we found that feeding a physiological level of dietary long-chain n-3 fatty acids did not alter IL-2 production by Con A-stimulated splenocytes (Figure 4.3) although splenocyte proliferative response to Con A, as determined by [$^3$H]-thymidine incorporation, was significantly suppressed (Figure 4.1). Consistent with other reports (Jolly et al 1997), long-chain n-3 fatty acids did not alter the proportion of T cells (CD4$^+$ or CD8$^+$) in spleen (Table 4.7), suggesting that n-3 fatty acids suppress Con A-induced cell proliferation by a mechanism other than a reduction in T cells. Con A is a lectin which binds specifically to certain sugar residues on T cell surface glycoproteins, including the T cell receptor and CD3, thereby stimulating T cells (Fleischer 1984). It has been proposed that dietary fish oil-induced modifications in membrane C20:4(6) impairs Con A binding and signal transduction (Valette et al. 1991). Although C20:4(6) levels in immune cell membrane phospholipids were reduced in high n-3-fed rats, our results suggest that dietary long-chain n-3 fatty acids do not interfere with early Con A stimulation events since IL-2 was still produced in high n-3-fed rats and was not different from those fed the low n-3 diet (Figure 4.3). However, diet-induced changes in the fatty acid composition of phospholipids may inhibit later membrane-associated events along the T cell activation pathway since Con A-induced cell proliferation was inhibited in rats fed the high n-3 diet. These findings should further be considered in association with post-culture changes in membrane composition since the phospholipid composition of Con A-activated cells from high n-3-fed rats was different from that of low n-3-fed rats. It is possible that these membrane changes could have affected cell proliferation or vice versa.

We also stimulated immune cells with a combination of PMA, which activates protein kinase C, and Ionomycin, a $Ca^{2+}$ ionophore which increases intracellular calcium concentration (Berry et al. 1989, Truneh et al. 1985). Activation of immune cells in this manner bypasses the cell membrane and involvement of the T cell receptor and CD3. In contrast to our results using Con A, cell proliferation was not significantly affected by dietary long-chain n-3 fatty acids when splenocytes were stimulated with PMA plus Ionomycin (Figure 4.2). This suggests that a membrane-associated event, prior to stimulation of protein kinase C and the rise in intracelluar calcium, may be altered in rats fed long-chain n-3 fatty acids. In contrast to Con A (primarily a T cell mitogen), PMA plus Ionomycin stimulates most cell types and further work investigating the role of long-chain n-3 fatty acids in altering the function of antigen presenting cells (B cells and macrophages) is needed.

To our surprise, the sex of the rat influenced the proportion of T cells ($CD4^+$) in spleen, but only in the low n-3 diet group (Table 4.7). Sex also influenced the IL-2 response to Con A and to PMA plus Ionomycin in low n-3-fed rats (Figure 4.3). Sex differences in immune responses have been previously reported in Fischer 344 rats (Davila and Kelley 1988). However, it is not known why sex-associated immune differences differed with dietary fat composition and this requires further study.

Overall, we have shown that feeding rats fish oil-derived long chain n-3 fatty acids at a level achievable in the human diet (5 g/100 g total fat) alters the lipid composition of resting and activated immune cells and also affects membrane-mediated immune functions, such as NK cell cytotoxicity and T cell proliferation. Unlike many previous studies which have concluded that a high intake of fish oil is immunosuppressive, we found that long-chain n-3 fatty acids upregulated NK cell cytotoxicity, a component of the innate immune system. However, T cell proliferation in response to Con A was suppressed by long-chain n-3 fatty acids in our healthy rat model. Our results suggest that different components of the immune system respond differently to a physiological level of long-chain n-3 fatty acids in the diet. It is possible that diet-induced changes in immune cell membrane phospholipids which are maintained in an activated state have diverse effects on membrane-associated events involved in the NK cell cytotoxicity and T cell activation pathways.

**TABLE 4.1 Fatty Acid Composition of Low and High n-3 Diets Fed to Rats[1]**

| | Low n-3 Diet | High n-3 Diet |
|---|---|---|
| **Fatty acid** | % w/w | |
| C14:0 | 1.3 | 2.0 |
| C16:0 | 15.7 | 15.7 |
| C18:0 | 26.9 | 25.5 |
| C18:1 (9) | 8.4 | 8.2 |
| C18:2 (6) | 44.3 | 39.3 |
| C18:3 (3) | 1.2 | 0.2 |
| C20:5 (3) | nd | 3.2 |
| C22:5 (3) | nd | 0.2 |
| C22:6 (3) | nd | 0.8 |
| SFA | 45.6 | 44.8 |
| MUFA | 8.7 | 10.4 |
| PUFA | 45.3 | 44.5 |
| n-6 PUFA | 44.3 | 39.6 |
| n-3 PUFA | 1.2 | 5.1 |
| n-6/n-3 ratio | 37 | 7.8 |
| P/S ratio | 1.0 | 1.0 |

[1] Values are g/100 g of total fat, except for ratios. Diets contained 200 g/kg of fat from a mixture of sources [safflower oil, hard beef tallow, linseed oil (low n-3 diet only), and fish oil (high n-3 diet only]. Minor fatty acids are not reported, therefore totals do not add up to 100%. Abbreviations used: nd, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids.

**TABLE 4.2 Monoclonal Antibodies Used in Indirect Immunofluorescence (Phenotype) Assay**

| Clone[1] | Specificity | Description |
|---|---|---|
| OX19 | CD5 | Reacts with 69 kDa surface glycoprotein on thymocytes and peripheral T cells |
| w3/25 | CD4 | Reacts with 48-53 kDa surface glycoprotein on T helper cells and peritoneal macrophages |
| OX8 | CD8a | Reacts with 34 kDa α chain of CD8 antigen on thymocytes, T suppressor/cytotoxic cells, natural killer (NK cells), and some activated T helper cells |
| OX12 | Ig κ chain | Reacts with immunoglobulin (Ig) κ chain on B cells |
| OX42 | CD11b/c | Reacts with 160, 103, and 95 kDa cell surface proteins on most resident peritoneal and activated macrophages, granulocytes, monocytes, and dendritic cells |
| 3.2.3 | CD161 | Reacts with NKR-P1A on NK cells and subset of T cells |

[1] All monoclonal antibodies were kindly provided by Dr. A. Rabinovitch (University of Alberta, Edmonton, AB, Canada) with the exception of 3.2.3 (Cedarlane Laboratories Ltd., Hornby, ON, Canada). All mAb were mouse anti-rat IgG.

TABLE 4.3 Effect of Diet and Sex on Food Intake, Body Weight and Spleen Weight of Rats[1]

| | **Low n-3 Diet** | | **High n-3 Diet** | | **Significance**[2] | |
|---|---|---|---|---|---|---|
| | Male ($n = 4$) | Female ($n = 4$) | Male ($n = 4$) | Female ($n = 4$) | Sex, p < | Diet, p < |
| Food Intake, g/kg body weight/d | 60 ± 4 | 60 ± 3 | 63 ± 5 | 58 ± 2 | NS | NS |
| Final body weight, g | 226 ± 13 | 152 ± 6 | 234 ± 18 | 156 ± 5 | 0.0001 | NS |
| Weight increase, g | 38 ± 6 | 14 ± 2 | 43 ± 6 | 9 ± 2 | 0.0001 | NS |
| Spleen weight, mg | 537 ± 27 | 417 ± 21 | 590 ± 33 | 449 ± 21 | 0.0003 | NS |
| Spleen weight, g/ kg body weight | 2.4 ± 0.1 | 2.8 ± 0.2 | 2.5 ± 0.1 | 2.9 ± 0.1 | 0.02 | NS |
| Spleen cells (x $10^6$)/g spleen | 459 ± 19 | 390 ± 28 | 459 ± 54 | 414 ± 14 | NS | NS |

[1] Values are means ± SEM for male and female rats within low and high n-3 diet groups. *n* = number of rats per group.

[2] Significant effect of sex and diet as determined by two-way ANOVA, NS = $p > 0.05$. No significant interactions were found.

TABLE 4.4 Effect of Diet and Cell Culture Condition on Fatty Acid Composition of Phosphatidylcholine in Rat Splenocytes[1]

| | Freshly isolated | | | 48 h (- Con A) | | | 48 h (+ Con A) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | Low n-3 | High n-3 | Diet p <[2] | Low n-3 | High n-3 | Diet p < | Low n-3 | High n-3 | Diet p < | Culture p <[3] | Con A p <[4] |
| C18:2 (6) | 12 ± 0.4 | 17 ± 1 | 0.001 | 6.0 ± 0.6 | 8.0 ± 0.5 | 0.01 | 5.9 ± 0.4 | 7.7 ± 0.4 | 0.005 | 0.0001 | NS |
| C20:4 (6) | 22 ± 1 | 14 ± 1 | 0.001 | 18 ± 1† | 12 ± 1 | 0.001 | 11 ± 1 | 8.1 ± 0.7 | 0.05 | 0.005 | 0.0001 |
| C20:5 (3) | 0.20 ± 0.09 | 1.0 ± 0.1 | 0.001 | 0.42 ± 0.21 | 1.6 ± 0.4 | 0.01 | nd[5] | 0.56 ± 0.10 | 0.001 | NS | 0.002 |
| C22:4 (6) | 1.8 ± 0.1 | 0.67 ± 0.04 | 0.001 | 1.5 ± 0.4 | 1.1 ± 0.1 | NS | 1.2 ± 0.3 | 0.95 ± 0.15 | NS | NS | NS |
| C22:5 (6) | 0.30 ± 0.02 | nd | 0.001 | 0.41 ± 0.18 | 0.42 ± 0.18 | NS | 0.31 ± 0.12 | nd | 0.05 | NS | NS |
| C22:5 (3) | 0.47 ± 0.06 | 1.8 ± 0.1 | 0.001 | 0.92 ± 0.12 | 1.8 ± 0.3 | 0.01 | 0.55 ± 0.10 | 1.6 ± 0.1 | 0.001 | NS | NS |
| C22:6 (3) | 0.75 ± 0.07 | 1.7 ± 0.1 | 0.001 | 0.90 ± 0.10 | 1.4 ± 0.1† | 0.01 | 1.0 ± 0.1 | 1.6 ± 0.1 | 0.001 | NS | NS |
| SFA | 46 ± 1 | 50 ± 1 | 0.001 | 52 ± 2 | 51 ± 2 | NS | 50 ± 1 | 48 ± 2 | NS | 0.03 | 0.03 |
| MUFA | 12 ± 1 | 13 ± 1 | NS | 17 ± 1 | 18 ± 1 | NS | 25 ± 2 | 26 ± 2 | NS | 0.0001 | 0.0001 |
| n-6 PUFA | 39 ± 1 | 34 ± 1 | 0.005 | 31 ± 1 | 26 ± 1 | 0.01 | 22 ± 2 | 21 ± 1 | NS | 0.0001 | 0.0001 |
| n-3 PUFA | 1.5 ± 0.2 | 4.4 ± 0.2 | 0.001 | 2.4 ± 0.3 | 4.8 ± 0.7 | 0.001 | 1.5 ± 0.2 | 3.6 ± 0.2 | 0.001 | NS | 0.01 |
| n-6/n-3 | 34 ± 3 | 8.3 ± 0.9 | 0.001 | 14 ± 1† | 5.9 ± 0.8 | 0.001 | 15 ± 2 | 5.6 ± 0.2 | 0.001 | 0.0001 | NS |
| P/S | 0.89 ± 0.03 | 0.74 ± 0.03 | 0.01 | 0.61 ± 0.06 | 0.64 ± 0.06 | NS | 0.49 ± 0.05 | 0.52 ± 0.04 | NS | 0.0005 | NS |

[1] Values are percentages of total fatty acids (except for ratios) and are expressed as means ± SEM ($n \geq 6$ per diet).

[2] Diet differences within each culture condition were determined by one-way ANOVA, NS = $p > 0.05$.

[3] Differences between freshly isolated splenocytes and splenocytes cultured without Con A for 48 h were determined by two-way ANOVA followed by a Duncan's multiple range test. When an interaction was found † indicates diet for which there was a significant effect of culture, NS = $p > 0.05$.

[4] Differences between splenocytes cultured without Con A for 48 h and splenocytes cultured with Con A for 48 h were determined by two-way ANOVA. NS = $p > 0.05$.

[5] nd = not detectable

**TABLE 4.5 Effect of Diet and Cell Culture Condition on Fatty Acid Composition of Phosphatidylethanolamine in Rat Splenocytes[1]**

| | Freshly isolated | | | 48 h (- Con A) | | | 48 h (+ Con A) | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | Low n-3 | High n-3 | Diet p <[2] | Low n-3 | High n-3 | Diet p < | Low n-3 | High n-3 | Diet p < | Culture p <[3] | Con A p <[4] |
| C18:2 (6) | 4.5 ± 0.1 | 5.9 ± 0.3 | 0.01 | 2.2 ± 0.1 | 2.8 ± 0.3 | NS | 2.6 ± 0.3 | 3.4 ± 0.2 | 0.04 | 0.0001 | 0.02 |
| C20:4 (6) | 37 ± 2 | 32 ± 1 | 0.05 | 25 ± 1 | 23 ± 2 | NS | 26 ± 2 | 25 ± 2 | NS | 0.0001 | NS |
| C20:5 (3) | 0.18 ± 0.06 | 2.8 ± 0.1 | 0.001 | 0.34 ± 0.18 | 2.2 ± 0.6 | 0.02 | 0.29 ± 0.10 | 1.2 ± 0.2 | 0.003 | NS | NS |
| C22:4 (6) | 7.9 ± 0.6 | 2.6 ± 0.1 | 0.001 | 9.5 ± 0.3 | 3.7 ± 0.9 | 0.001 | 10 ± 1 | 5.4 ± 0.7 | 0.003 | 0.02 | NS |
| C22:5 (6) | 1.2 ± 0.1 | 0.12 ± 0.02 | 0.001 | 4.5 ± 1.0 | 1.5 ± 0.9 | 0.05 | 1.1 ± 0.2 | 0.39 ± 0.18 | 0.04 | 0.001 | 0.01 |
| C22:5 (3) | 1.4 ± 0.1 | 8.4 ± 0.3 | 0.001 | 1.8 ± 0.1 | 5.0 ± 0.8$^{\dagger}$ | 0.002 | 2.1 ± 0.2 | 6.8 ± 0.4$^{*}$ | 0.001 | 0.001 | 0.04 |
| C22:6 (3) | 2.6 ± 0.1 | 7.2 ± 0.4 | 0.001 | 1.9 ± 0.1 | 3.6 ± 0.5$^{\dagger}$ | 0.01 | 2.8 ± 0.2 | 6.0 ± 0.3$^{*}$ | 0.001 | 0.0001 | 0.0001 |
| SFA | 34 ± 2 | 31 ± 1 | NS | 40 ± 1 | 42 ± 3 | NS | 34 ± 2 | 32 ± 1 | NS | 0.0001 | 0.001 |
| MUFA | 9.0 ± 1.1 | 8.0 ± 0.7 | NS | 15 ± 1 | 16 ± 1 | NS | 15 ± 2 | 13 ± 1 | NS | 0.0001 | NS |
| n-6 PUFA | 53 ± 2 | 42 ± 1 | 0.001 | 43 ± 1 | 31 ± 1 | 0.01 | 41 ± 4 | 35 ± 3 | NS | 0.0001 | NS |
| n-3 PUFA | 4.8 ± 0.3 | 18 ± 1 | 0.001 | 4.3 ± 0.3 | 11 ± 1$^{\dagger}$ | 0.001 | 5.0 ± 0.4 | 14 ± 1$^{*}$ | 0.001 | 0.0001 | 0.001 |
| n-6/n-3 | 12 ± 1 | 2.3 ± 0.1 | 0.001 | 9.8 ± 0.8$^{\dagger}$ | 3.0 ± 0.1 | 0.001 | 7.5 ± 0.3$^{*}$ | 2.4 ± 0.1 | 0.001 | NS | 0.001 |
| P/S | 2.0 ± 0.2 | 1.9 ± 0.1 | NS | 1.1 ± 0.1 | 1.1 ± 0.2 | NS | 1.4 ± 0.2 | 1.6 ± 0.1 | NS | 0.0001 | 0.02 |

[1] Values are percentages of total fatty acids (except for ratios) and are expressed as means ± SEM ($n \geq 6$ per diet).

[2] Diet differences within each culture condition were determined by one-way ANOVA, NS = $p > 0.05$.

[3] Differences between freshly isolated splenocytes and splenocytes cultured without Con A for 48 h were determined by two-way ANOVA followed by a Duncan's multiple range test. When an interaction was found $^{\dagger}$ indicates diet for which there was a significant effect of culture, NS = $p > 0.05$.

[4] Differences between splenocytes cultured without Con A for 48 h and splenocytes cultured with Con A for 48 h were determined by two-way ANOVA followed by a Duncan's multiple range test. When an interaction was found * indicates diet for which there was a significant effect of Con A stimulation, NS = $p > 0.05$.

**TABLE 4.6 Effect of Diet and Cell Culture Condition on Fatty Acid Composition of Phosphatidylinositol in Rat Splenocytes**[1]

|  | Freshly isolated |  |  | 48 h (- Con A) |  |  | 48 h (+ Con A) |  |  |  |  |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Fatty acid | Low n-3 | High n-3 | Diet $p <$[2] | Low n-3 | High n-3 | Diet $p <$ | Low n-3 | High n-3 | Diet $p <$ | Culture $p <$[3] | Con A $p <$[4] |
| C18:2 (6) | 3.4 ± 0.2 | 3.5 ± 0.5 | NS | 2.1 ± 0.2 | 2.4 ± 0.3 | NS | 2.0 ± 0.2 | 2.2 ± 0.2 | NS | 0.0003 | NS |
| C20:4 (6) | 35 ± 3 | 32 ± 1 | NS | 21 ± 2 | 18 ± 2 | NS | 19 ± 2 | 18 ± 1 | NS | 0.0001 | NS |
| C20:5 (3) | 0.18 ± 0.05 | 1.0 ± 0.1 | 0.001 | 0.82 ± 0.20† | 0.96 ± 0.07 | NS | 0.29 ± 0.06 | 0.50 ± 0.18 | NS | NS | 0.001 |
| C22:4 (6) | 1.8 ± 0.1 | 0.86 ± 0.01 | 0.001 | 0.37 ± 0.07 | 0.55 ± 0.09 | NS | 0.46 ± 0.07 | 0.54 ± 0.09 | NS | 0.0001 | NS |
| C22:5 (6) | 0.15 ± 0.01 | nd | 0.001 | 2.3 ± 0.4 | 1.2 ± 0.2 | 0.04 | 3.4 ± 0.4 | 2.1 ± 0.5 | NS | 0.0001 | 0.03 |
| C22:5 (3) | 0.27 ± 0.04 | 1.7 ± 0.1 | 0.001 | 0.25 ± 0.07 | 0.81± 0.16† | 0.01 | 0.48 ± 0.07 | 1.5 ± 0.2 | 0.0001 | 0.0003 | 0.002 |
| C22:6 (3) | 0.37 ± 0.06 | 0.67 ± 0.06 | 0.01 | 0.17 ± 0.09 | 0.50 ± 0.08 | 0.02 | 0.51 ± 0.06 | 1.0 ± 0.1 | 0.0001 | 0.02 | 0.0001 |
| SFA | 49 ± 2 | 47 ± 1 | NS | 47 ± 3 | 47 ± 2 | NS | 49 ± 2 | 49 ± 3 | NS | NS | NS |
| MUFA | 5.4 ± 0.2 | 7.5 ± 0.7 | 0.02 | 11 ± 1 | 13 ± 1 | NS | 14 ± 1 | 13 ± 1 | NS | 0.0001 | NS |
| n-6 PUFA | 42 ± 3 | 38 ± 1 | NS | 28 ± 3 | 26 ± 3 | NS | 28 ± 2 | 26 ± 2 | NS | 0.0001 | NS |
| n-3 PUFA | 0.98 ± 0.07 | 3.7 ± 0.2 | 0.001 | 1.5 ± 0.3 | 2.5 ± 0.4† | 0.05 | 1.5 ± 0.1 | 2.8 ± 0.1 | 0.0001 | NS | NS |
| n-6/n-3 | 44 ± 5 | 11 ± 1 | 0.001 | 19 ± 3† | 9.8 ± 1.5 | 0.01 | 18 ± 2 | 10 ± 0.5 | 0.02 | 0.0002 | NS |
| P/S | 0.90 ± 0.09 | 0.91 ± 0.03 | NS | 0.65 ± 0.08 | 0.63 ± 0.09 | NS | 0.66 ± 0.08 | 0.63 ± 0.06 | NS | 0.007 | NS |

[1] Values are percentages of total fatty acids (except for ratios) and are expressed as means ± SEM ($n \geq 6$ per diet).

[2] Diet differences within each culture condition were determined by one-way ANOVA, NS = $p > 0.05$.

[3] Differences between freshly isolated splenocytes and splenocytes cultured without Con A for 48 h were determined by two-way ANOVA. When an interaction was found † indicates diet for which there was a significant effect of culture. NS = $p > 0.05$.

[4] Differences between splenocytes cultured without Con A for 48 h and splenocytes cultured with Con A for 48 h were determined by two-way ANOVA.

[5] nd = not detected

**TABLE 4.7 Effect of Long-Chain n-3 Fatty Acids on Immune Cell Phenotypes in Spleen of Male and Female Rats**[1]

| Immune Cell Phenotype | Low n-3 Diet | | High n-3 Diet | |
|---|---|---|---|---|
| | **Male** | **Female** | **Male** | **Female** |
| | % of total immune cells in spleen | | | |
| $CD5^+$ T cells | 43 ± 2 | 49 ± 2* | 44 ± 1 | 48 ± 1 |
| $CD4^+$ T helper cells | 24 ± 1 | 28 ± 1* | 26 ± 0.4 | 28 ± 1 |
| $CD8^+$ T suppressor/cytotoxic cells | 21 ± 1 | 23 ± 1 | 23 ± 1 | 23 ± 1 |
| B cells | 33 ± 2 | 33 ± 1 | 36 ± 2 | 33 ± 1 |
| Macrophages | 2.6 ± 0.6 | 3.0 ± 0.6 | 4.6 ± 1.5 | 3.1 ± 0.7 |
| Natural killer cells | 4.0 ± 1.5 | 5.2 ± 0.9 | 5.6 ± 0.7 | 5.0 ± 0.3 |
| CD4/CD8 | 1.2 ± 0.1 | 1.2 ± 0.1 | 1.2 ± 0.1 | 1.2 ± 0.1 |

[1] Values are means ± SEM, $n \geq 6$/diet. There were no significant effects of diet. * indicates significant effect of sex in the low n-3 diet group ($p < 0.05$). There were no other sex effects.

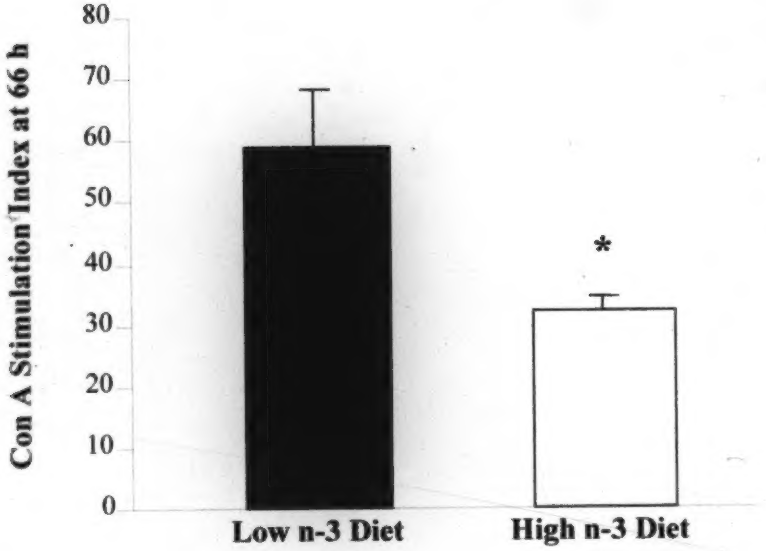

**FIGURE 4.1 Effect of Dietary Long-Chain n-3 Fatty Acids on Splenocyte Proliferative Response to Concanavalin A at 66 h.** Splenocytes were cultured with Concanavalin A (Con A, 5 mg/L) for 66 h as described in Materials and Methods. Mitogenic response is expressed as the stimulation index = [amount of [$^3$H]-thymidine (kBq/min) incorporated by stimulated cells - amount of [$^3$H]-thymidine (kBq/min) incorporated by unstimulated cells]/amount of [$^3$H]-thymidine (kBq/min) incorporated by unstimulated cells. Bars represent means ± SEM ($n \geq 5$/group). Sex did not affect the Con A stimulation index; therefore, male and female rats were combined within each diet group. * denotes a significant ($p < 0.03$) effect of diet as determined by Student's *t*-test.

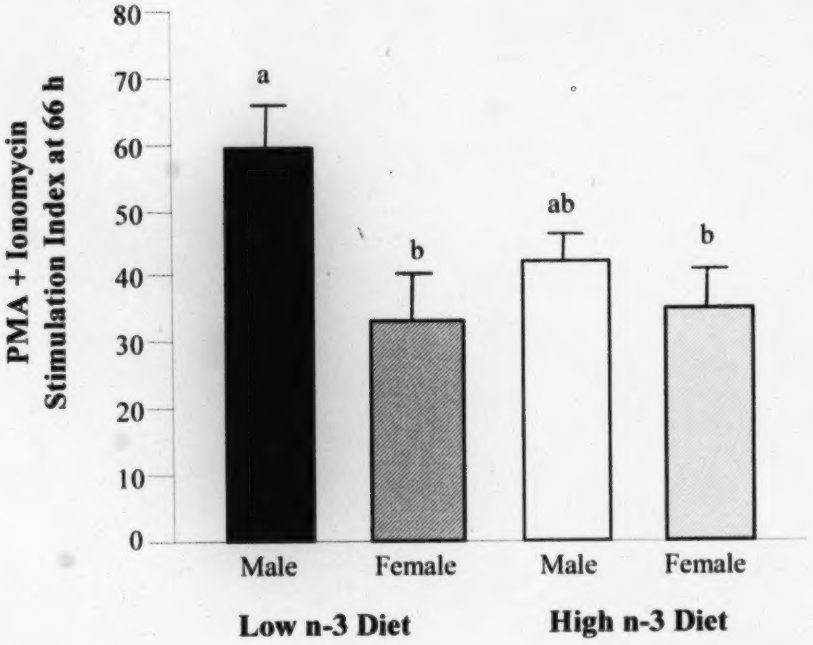

**FIGURE 4.2 Effect of Sex and Diet on Splenocyte Proliferative Response to Phorbol Myristate Acetate Plus Ionomycin at 66 h.** Splenocytes were cultured with Phorbol Myristate Acetate (PMA, 30 μg/L) plus Ionomycin (0.75 μmol/L) for 66 h as described in Materials and Methods. Mitogenic response is expressed as the stimulation index = [amount of [$^3$H]-thymidine (kBq/min) incorporated by stimulated cells - amount of [$^3$H]-thymidine (kBq/min) incorporated by unstimulated cells]/amount of [$^3$H]-thymidine (kBq/min) incorporated by unstimulated cells. Bars represent means ± SEM (*n* = 4/group). Bars that do not share a common letter are significantly different ($p < 0.05$) as determined by a Duncan's multiple range test.

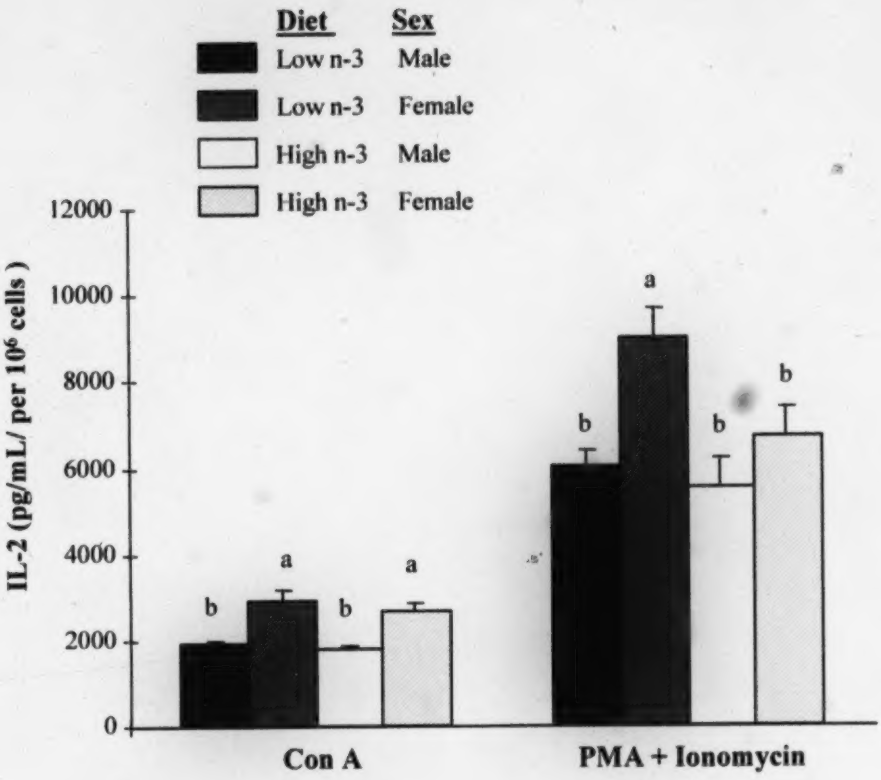

**FIGURE 4.3 Effect of Sex and Diet on Splenocyte Interleukin-2 Production.** Splenocytes were cultured without mitogen (unstimulated) or with Concanavalin A (Con A, 5 mg/L) or Phorbol Myristate Acetate (PMA, 30 μg/L) plus Ionomycin (0.75 μmol/L) for 48 h. The concentration of IL-2 in splenocytes culture supernatants was determined by enzyme-linked immunosorbent assay as described in Materials and Methods. For all groups, unstimulated splenocytes produced undectable levels of IL-2 in the culture supernatant. Bars represent means ± SEM (*n* ≥ 7/group). The effects of sex and diet were analyzed by two-way ANOVA. For each culture condition, bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

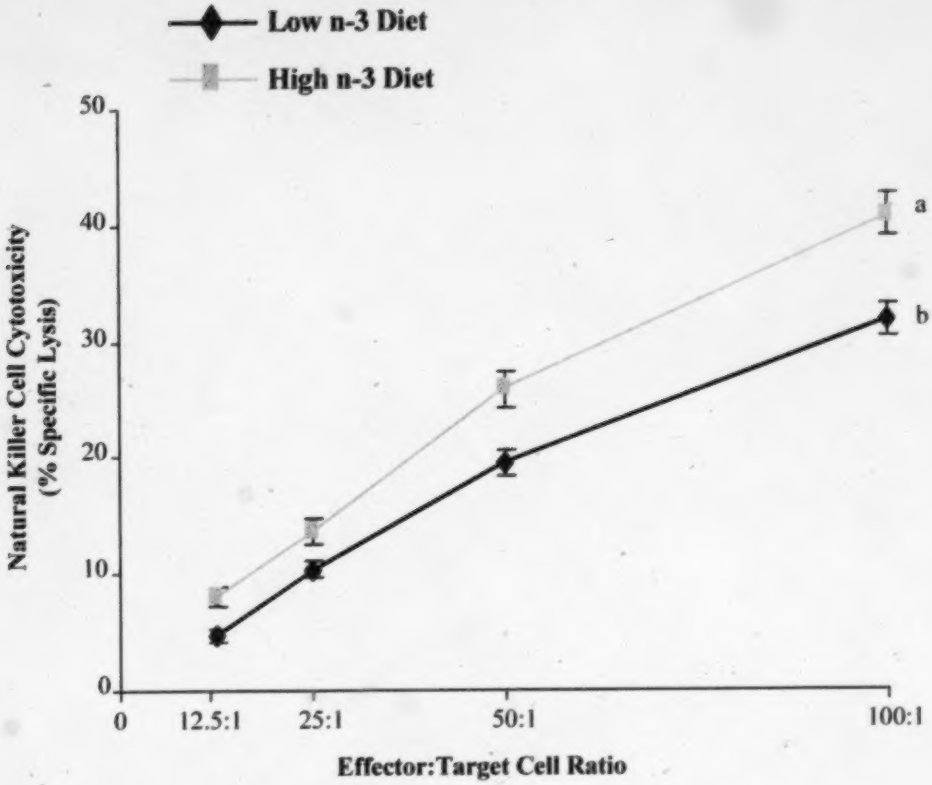

**FIGURE 4.4 Effect of Long-Chain n-3 Fatty Acids on Splenocyte Natural Killer Cell Cytotoxic Activity.** Natural killer (NK) cell cytotoxic activity is expressed as % specific lysis, which is equal to 100 x (mean experimental $^{51}$Cr release from labeled YAC-1 cells – mean spontaneous $^{51}$Cr release)/(mean maximum $^{51}$Cr release – mean spontaneous $^{51}$Cr release). Sex did not affect NK cell cytotoxicity; therefore, male and female rats were combined within each diet group. Points represent means ± SEM (*n* = 6/group). Lines are significantly different ($p < 0.05$) as determined by a one-way split-plot (repeated measures) ANOVA.

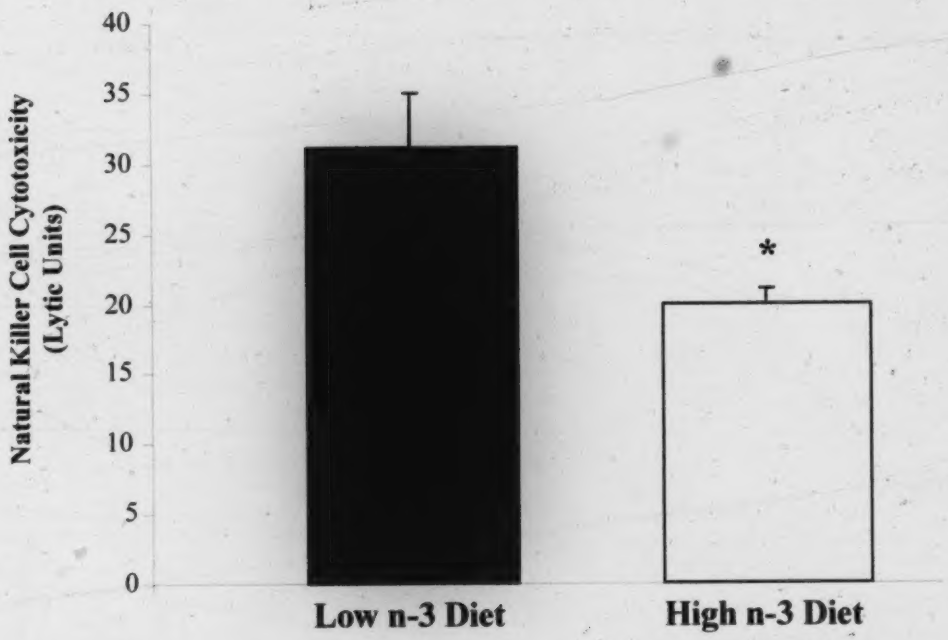

**FIGURE 4.5 Effect of Long-Chain n-3 Fatty Acids on Splenocyte Natural Killer Cell Lytic Activity.** Lytic units are defined as the number (x $10^{-3}$) of natural killer (NK) cells required to cause 20% lysis of target cells. Values are means ± SEM (*n* = 6/group). * denotes a significant ($p < 0.04$) effect of diet as determined by Student's *t*-test.

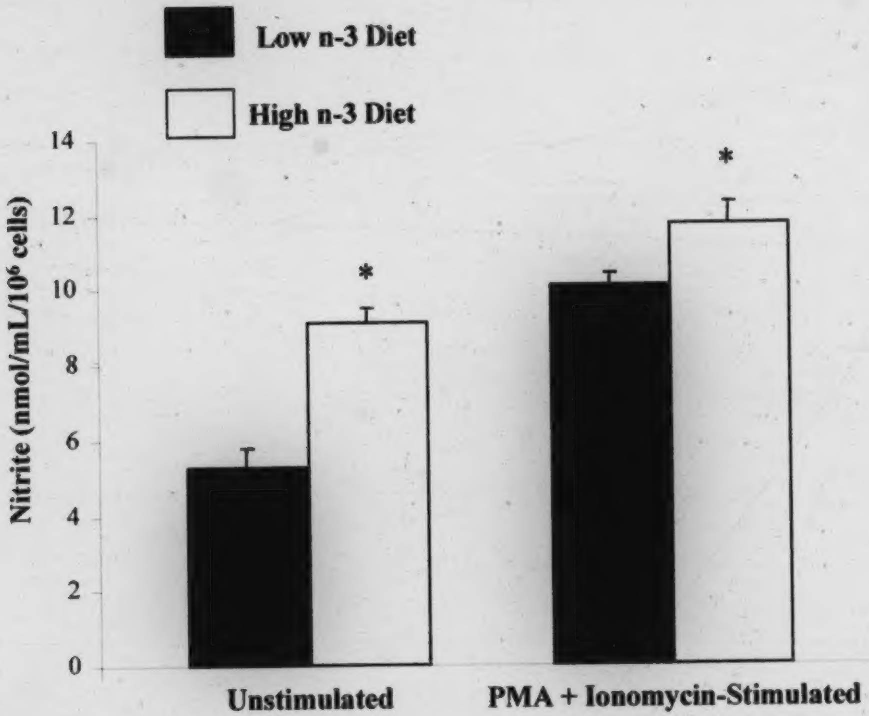

**FIGURE 4.6 Effect of Diet on Splenocyte Nitric Oxide Production.** Splenocytes were cultured without mitogen (unstimulated) or with Phorbol Myristate Acetate (PMA, 30 µg/L) plus Ionomycin (0.75 µmol/L) for 48 h. The concentration of nitrite ($NO_2$-) in cell culture supernatants was determined by the Griess reaction as described in Materials and Methods. Bars represent means ± SEM (*n* ≥ 7/group). Sex did not affect nitrite production; therefore, male and female rats were combined within each diet group. For each culture condition * denotes a significant ($p < 0.05$) effect of diet as determined by Student's *t*-test.

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# 5. THE ROLE OF DIETARY LONG-CHAIN N-3 FATTY ACIDS IN ANTICANCER IMMUNE DEFENSE AND R3230AC MAMMARY TUMOUR GROWTH IN RATS: INFLUENCE OF DIET FAT COMPOSITION[1,2]

## 5.1 INTRODUCTION

High levels of fish oil-derived long-chain n-3 fatty acids, eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids, inhibit the growth of certain carcinogen-induced and transplantable animal tumours (Ip 1997). It has been suggested that the ratio of n-6 to n-3 fatty acids in the diet, not the absolute levels of fatty acids, is critical for this effect (Calviello et al. 1998, Cohen et al. 1993, Abou-El-Ela et al. 1989, Karmali 1987). A dietary polyunsaturated to saturated fatty acid (P/S) ratio of 1 is currently recommended by several health agencies (American Diabetes Association 1999, Krauss et al. 1996, Health and Welfare Canada 1990). However, the observation that high levels of n-6 polyunsaturated fatty acids promote tumour growth in animals (Ip 1997) has likely contributed to the lack of a similar recommendation by cancer agencies (World Cancer Research Fund 1997).

The mechanism for the putative anticancer benefits of dietary long-chain n-3 fatty acids is not known. We investigated whether the tumour suppressor effect of long-chain n-3 fatty acids may occur, in part, via alterations in the host immune system. Diets rich in EPA and DHA can affect both the innate (natural) and acquired (specific) cell-mediated immune systems (Calder 1998). Cellular components of both of these immune systems, including natural killer (NK) cells, macrophages, $CD4^+$ T helper and $CD8^+$ T suppressor/cytotoxic cells, are involved in anticancer immunity (Whiteside and Herberman 1995, Robins 1986, Adams et al. 1982). Feeding healthy rats long-chain n-3

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[1] A version of this chapter has been submitted for publication to *Nutrition and Cancer*.

[2] This work was presented in part at the International Congress of Nutrition, July 1997, Montréal, Quebec (Robinson, L. E. and Field, C. J. Supplementation of a high polyunsaturated diet with long chain n-3 fatty acids does not inhibit R3230AC mammary tumour growth in rats). Also presented in part at Experimental Biology, April 1998, San Francisco, CA (Robinson, L. E. and Field, C. J. Long chain n-3 fatty acids fed in a low polyunsaturated diet improve membrane-mediated anti-tumour immune defenses and reduce tumour growth) and Experimental Biology, April 1999, Washington, DC (Robinson, L. E. and Field, C. J. Influence of diet fat composition on incorporation of long-chain n-3 fatty acids into R3230AC tumour cell phospholipids).

fatty acids at a level which could potentially be achieved in the human diet (5 % w/w of total fat) significantly enhanced NK cell cytotoxicity and the proportion of activated immune cells (Chapter 3).

A direct effect of dietary lipids on modulation of tumour cell membrane composition may also play a role in the ability of EPA and DHA to inhibit tumour growth (Jiang et al. 1998). It is well established that changes in dietary fat composition can induce significant alterations in the composition and function of membranes in a variety of human and animal tissues (Field et al. 1988, Clandinin et al. 1985), including tumours (Calviello et al. 1998, Rose et al. 1995, Jenski et al. 1993, Karmali et al. 1984). For example, incorporation of long-chain n-3 fatty acids into tumour cell membranes may alter membrane-mediated functions involved in tumour growth inhibition (Calviello et al. 1998), susceptibility to attack by the host immune system (Jenski et al. 1993) and response to chemotherapy (de Salis and Meckling-Gill 1995).

The present study was performed as two experiments using the R3230AC transplantable mammary adenocarcinoma in rats. The objective of Experiment 1 was to determine if fish oil-derived long-chain n-3 fatty acids improve antitumour immune defense and inhibit tumour growth when fed as part of a high polyunsaturated fat diet, representative of that currently recommended by several health agencies for the human population. We found no significant effects of dietary long-chain n-3 fatty acids on tumour growth or host immune responses in Experiment 1. Thus, we performed a second experiment to determine if the addition of long-chain n-3 fatty acids to a low polyunsaturated fat diet, representative of that currently consumed by a large segment of the North American population, would enhance immune defense and suppress tumour growth. The effect of dietary EPA and DHA on individual phospholipid fractions in tumour cell membranes was also studied in both experiments.

## 5.2 MATERIALS AND METHODS

**Animals, diets, and tumour implantation.** Experiments were reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Forty-seven female Fischer 344 rats (143 ± 1 g) were obtained from a colony maintained at the University of Alberta and were housed in individual wire-mesh cages in a temperature controlled room (23 °C) maintained on a 12 h light/dark cycle. Body weight and food intake were recorded every second day in Experiment 1 and every third day in Experiment 2.

Rats were randomly assigned to be fed nutritionally complete, semi-purified diets (Teklad Test Diets, Madison, WI) containing (per kg) 270 g high protein casein, 408 g carbohydrate and 200 g fat. The complete nutrient composition of the diets was described in Chapter 3. In Experiment 1, diets had a P/S ratio of 1 (high P/S diet), while in Experiment 2 the P/S ratio was 0.35 (low P/S diet) as determined by gas-liquid chromatography (Field et al. 1988). At each P/S ratio there were two diets which differed only in the composition of fat, providing two different levels of long chain n-3 fatty acids from a mixed fish oil source (P-28 Nisshin lot # 28020, Nisshin Flour Milling Co., Ltd., Tokyo, Japan): low (0 g/kg) or high (51 g/kg of total fat). The only source of n-3 fatty acids in the low n-3 diet was α-linolenic acid (C18:3n-3), provided by linseed oil (Galaxy Enterprises, Edmonton, Canada). The fatty acid composition of the diets is presented in **Table 5.1**. All animals were given free access to food and water.

R3230AC mammary adenocarcinoma cells were obtained from the DCT Tumour Repository (National Cancer Institute, Frederick, MD) and were maintained in our laboratory as frozen stocks. Previously frozen R3230AC tumour cells were transplanted into animals used in the study after 3 passages in recipient animals of the same age, sex, and strain. Specifically, after 21 d of dietary treatment, a freshly harvested R3230AC mammary tumour from a rat implanted 2-3 wk earlier was finely chopped under sterile conditions to prepare a tumour brei and 50 μL was injected subcutaneously in the inguinal region of experimental rats. Rats were killed by $CO_2$ asphyxiation and cervical dislocation 17 d following tumour implantation. At necropsy, tumour and spleen were removed and weighed to be used for the measurements described below. In Experiment 1 (high P/S diet), 14 rats (7/diet) were implanted with the tumour. In Experiment 2 (low P/S diet), there were 14 tumour-bearing rats (7/diet) and 19 healthy (control) rats (9 rats were fed the low n-3 diet and 10 rats were fed the high n-3 diet). Due to the number of variables being measured, this study was carried out in a series of replicate experiments, such that all measurements were not performed on individual animals. For each assay, the number of animals in each treatment group is provided in the text, table, or figure legend.

**Splenocyte isolation and activation.** Splenocytes were isolated aseptically in Krebs-Ringer HEPES buffer (KRH, pH 7.4) supplemented with bovine serum albumin (BSA, 5 g/L; Sigma Chemical, St. Louis, MO) as described in Chapter 3. Isolated splenocytes ($3.0 \times 10^9$ cells/L) in complete culture media (CCM; defined in Chapter 3) were incubated in 24-well sterile plates (Costar®, Corning Glass Works, Corning, NY) for 48 h in a humidified atmosphere at 37 °C in the presence of 5% $CO_2$. The cell culture media contained either no mitogen (unstimulated cells) or was supplemented with either Concanavalin A (Con A, 5 mg/L; ICN, Montréal, QB, Canada) or Phorbol Myristate Acetate (PMA, 30 μg/L; ICN) plus Ionomycin (0.75 μmol/L; Sigma Chemical). After 48 h, splenocytes were centrifuged at 228 g (Beckman J2-HC, Beckman Instruments, Palo Alto, CA) for 10 min at 4°C. Splenocyte culture supernatants were collected and stored at –70 °C for subsequent nitric oxide and cytokine analyses. Pelleted splenocytes (unstimulated and stimulated) were then washed twice in KRH (pH 7.4) supplemented with BSA (5 g/L) in preparation for indirect immunofluorescence analyses.

**Indirect immunofluorescence (phenotype) assay.** Immune cell subsets in freshly isolated splenocytes and mitogen-stimulated splenocytes were identified by indirect immunofluorescence assay as described in Chapter 3. The monoclonal antibodies used were the same as those defined in Chapter 3 with the exception of OX39 (Cedarlane Laboratories Ltd., Hornby, ON, Canada) and JJ319 (PharMingen, Mississauga, ON, Canada) (**Table 5.2**). OX39 recognizes the α-chain of the interleukin-2 (IL-2) receptor (CD25) on activated T and B cells and macrophages, while JJ319 reacts with a costimulatory receptor molecule (CD28) on T cells (Table 5.2).

**NK cell cytotoxicity assay.** A 4 h sodium chromate ($^{51}Cr$, Amersham, Oakville, ON, Canada) release assay was performed using NK cell-sensitive YAC-1 murine lymphoma cells (American Type Culture Collection, Rockville, MD) as targets and freshly isolated splenocytes as effector cells as described in Chapter 3.

**Nitric oxide production**. Nitric oxide production was determined by analyzing nitrite ($NO_2^-$, a product of the L-arginine-dependent nitric oxide pathway) concentration in splenocyte culture supernatants using a colorimetric assay based on the Griess reaction (Green et al. 1982) as described in Chapter 4.

**IL-2 production**. IL-2 concentration in culture supernatants collected from unstimulated and stimulated splenocytes was determined by a colorimetric enzyme-linked immunosorbent assay (ELISA) as described in Chapter 4.

**Tumour cell isolation.** At necropsy, R3230AC mammary tumours were removed and weighed. Tumour cells were isolated by a modification of the method described by Harmon and Hilf (1976). Non-necrotic, viable tumour tissue was finely chopped with scissors and suspended in 10 mL of cold Hanks' Balanced Salt Solution (HBSS; $Ca^{2+}$ and $Mg^{2+}$ free, pH 7.4, Appendix A) containing hyaluronidase (1 g/L; Sigma Chemical) and collagenase (type II, 0.5 g/L; Worthington Biochemical Corporation, Freehold, NJ). Flasks were covered and incubated in an orbital shaker (50 cycles/min) at 37 °C. After 60 min, the suspension was filtered through a wire strainer, pelleted (80 g for 5 min at 4°), resuspended in a fresh 10 mL of enzyme solution, and incubated for an additional 60 min in an orbital shaker (50 cycles/min). After incubation, the suspension was filtered through a mesh screen and pelleted (80 g for 5 min at 4°). The pellet was resuspended in 2 mL of HBSS supplemented with BSA (5 g/L) and separated by density gradient centrifugation using a 40% Percoll® (Pharmacia Biotech, Uppsala, Sweden) gradient (1800 rpm for 30 min at 4°). The cell interface was removed and washed 3 times in HBSS supplemented with BSA (5 g/L). Tumour cell viability was determined by trypan blue exclusion and was ≥ 90% for all treatment groups. Cell pellets were frozen at –70 °C for subsequent fatty acid analysis.

**Tumour cell fatty acid analysis.** Lipids were extracted from R3230AC mammary tumour cells by a modified Folch (Folch et al. 1957) procedure (Field et al. 1988) using the same method described for splenocytes in Chapter 3. Individual phospholipids were separated on Whatman HPK thin layer chromatography plates (10 x 10 cm) and compared with appropriate standards as described in Chapter 3. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI) fatty acid methyl esters were prepared using 14% (w/v) $BF_3$/methanol reagent (Morrison and Smith 1964) and separated by automated gas liquid chromatography as described in Chapter 3.

**Statistical analysis.** Results are presented as means ± SEM. All statistical analyses were conducted using the SAS statistical package (Version 6.11, SAS Institute, Cary, NC). In Experiment 1, the effect of diet was analyzed by Student's *t*-test. In Experiment 2, the main effects of diet and tumour were determined by two-way ANOVA followed by a Duncan's multiple range test to identify significant ($p \leq 0.05$) differences between individual treatments (Steel and Torie 1980). Body weight changes, food intake, and NK cell cytotoxicity were compared among groups by a split-plot (repeated measures) ANOVA (Steel and Torrie 1980). Paired *t* tests were used to compare $NO_2^-$ and IL-2 production by immune cells with or without mitogen. All tumour cell fatty acid analysis was done at the same time and the effects of long chain n-3 fatty acids and P/S ratio on phospholipid composition were compared by ANOVA and a Duncan's multiple range test.

## 5.3 RESULTS

**Food intake, body weight and spleen weight.** In the high P/S diet group, food intake was significantly lower on day 34 for low n-3-fed rats and day 36 for high n-3-fed rats when compared with food intake on day 20 (prior to tumour implantation) (**Figure 5.1**). In the low P/S diet group, food intake was not significantly affected by the tumour over the 17 d post-implantation period (**Figure 5.2**). Long-chain n-3 fatty acids did not significantly affect food intake throughout the experiment when supplemented in either the high P/S diet (Figure 5.1 and **Table 5.3**, mean food intake was 57 ± 1 g/kg body weight/d, *n* = 14;) or low P/S diet (Figure 5.2 and Table 5.3, mean food inake was 63 ± 1g/kg body weight/d, *n* = 29). Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect final body weight (154 ± 3 g, *n* = 14), weight increase (16 ± 2 g, *n* = 14), relative spleen weight (3.2 ± 0.1 g/kg body weight, *n* = 14), or the number of spleen cells (x $10^6$) isolated per gram of spleen (401 ± 27, *n* = 14) (Table 5.3). In the low P/S diet group, neither dietary long-chain n-3 fatty acids nor the tumor significantly affected final body weight (158 ± 2 g, *n* = 33), weight increase (13 ± 1 g, *n* = 33), relative spleen weight (2.8 ± 0.1 g/kg body weight, *n* = 33), or the number of spleen cells (x $10^6$) isolated per gram of spleen (433 ± 13, *n* = 32) (Table 5.3).

**R3230AC mammary tumour weight.** Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect final tumour weight (1.2 ± 0.1 g/100 g body weight, *n* = 14) (**Figure 5.3**). In the low P/S diet group, final tumour weight was lower when rats were fed long-chain n-3 fatty acids (1.3 ± 0.2 vs. 0.9 ± 0.1 g/100 g body weight, *n* = 7/diet), but this did not reach statistical significance (-31%, p = 0.1) (Figure 5.3).

### Immune cell phenotypes in freshly isolated splenocytes

**(a) High P/S Diet:** Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect the proportion (% of total) of total $CD5^+$ T cells (51 ± 1%), $CD4^+$ T helper cells (30 ± 1%), $CD8^+$ T suppressor/cytotoxic cells (18 ± 1%), B cells (30 ± 1%), macrophages (4.0 ± 0.4%), or the CD4/CD8 ratio (1.8 ± 0.1) in freshly isolated splenocytes (*n* = 14) (**Table 5.4**). However, rats fed the high n-3 diet had a significantly lower ($p < 0.01$) relative proportion of NK cells compared with those fed the low n-3 diet (Table 5.4). The relative proportion of freshly isolated splenocytes expressing the IL-2 receptor (CD25) was low (< 1%) and was not significantly affected by diet (Table 5.4).

**(b) Low P/S Diet:** Tumour burden did not significantly affect immune cell phenotypes in freshly isolated splenocytes with the following exception: in both diet groups, tumour-bearing rats had a significantly higher proportion of $CD8^+$ T suppressor/cytotoxic cells and a significantly lower proportion of B cells compared with healthy rats (**Table 5.5**). Feeding long-chain n-3 fatty acids in the low P/S diet did not significantly affect immune cell phenotypes in freshly isolated splenocytes with the following exception: both healthy and tumour-bearing rats fed the high n-3 diet had a significantly lower proportion of $CD5^+$ and $CD4^+$ T cells compared with those fed the low n-3 diet (Table 5.5). The relative proportion of freshly isolated splenocytes expressing either CD28 or the IL-2 receptor (CD25) was low (< 2%) and was not significantly different between treatment groups (Table 5.5).

### Immune cell phenotypes after 48 h Con A stimulation

**Low P/S Diet:** In both the low and high n-3 diet groups, tumour-bearing rats had a significantly higher proportion of $CD4^+$ T helper cells, $CD8^+$ T suppressor/cytotoxic cells and $CD28^+$ cells and a lower proportion of B cells following Con A stimulation compared with healthy rats (**Figure 5.4**). In addition, tumour-bearing rats fed the high n-3 diet had a significantly higher proportion of $CD4^+$, $CD8^+$ and $CD28^+$ cells and a lower proportion of B cells compared with those fed the low n-3 diet (Figure 5.4). Neither diet nor tumour significantly affected the relative proportion of macrophages ($3.6 \pm 0.3$ %, $n = 28$, Figure 5.4) or the CD4/CD8 ratio ($2.2 \pm 0.1$, $n = 29$) after Con A stimulation.

### IL-2 receptor (CD25) expression after 48 h Con A stimulation

**Low P/S Diet:** There was a significant interaction between long-chain n-3 fatty acids and the tumour on IL-2 receptor (CD25) expression on $CD5^+$ T cells after splenocytes were stimulated with Con A (**Table 5.6**). Tumour-bearing rats fed the high n-3 diet had a significantly higher proportion of $CD25^+$ $CD5^+$ total T cells compared with those fed the low n-3 diet, while diet did not affect the proportion of these cells in healthy rats (Table 5.6). In the high n-3 diet group, tumour-bearing rats had a significantly higher proportion of $CD5^+$ total T cells expressing CD25 compared with healthy rats, while the tumour did not affect the proportion of these cells in the low n-3 diet group (Table 5.6). In both the low and high n-3 diet groups, tumour-bearing rats had a significantly higher proportion of $CD25^+$ $CD4^+$ T helper cells and $CD25^+$ $CD28^+$ cells and a significantly lower proportion of B cells and macrophages that were $CD25^+$ compared with healthy rats (Table 5.6). Both healthy and tumour-bearing rats fed the high n-3 diet had a significantly higher proportion of $CD25^+$ $CD8^+$ T suppressor/cytotoxic cells and $CD25^+$ $CD28^+$ cells compared with those fed the low n-3 diet (Table 5.6).

**NK cell cytotoxicity**

**(a) High P/S Diet:** Feeding tumour-bearing rats long-chain n-3 fatty acids in the high P/S diet did not significantly affect NK cell cytotoxic activity at the 25:1 (19 ± 2%), 50:1 (29 ± 2%), and 100:1 (43 ± 2%) effector:target cell ratios ($n = 14$) (**Figure 5.5**). However, at the 12.5:1 effector:target cell ratio, the % specific lysis of target YAC-1 cells was significantly greater ($p < 0.01$) for splenocytes from rats fed the high n-3 diet relative to splenocytes from those fed the low n-3 diet (13 ± 1% vs 9.5 ± 0.4%, $n = 6$/diet, **Figure 5.6**). Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect lytic activity (13 ± 2 lytic units, $n = 14$).

**(b) Low P/S Diet:** There was a significant effect of long-chain n-3 fatty acids, tumour, and their interaction on NK cell cytotoxic activity when rats were fed the low P/S diet (**Figure 5.7**). At each effector:target cell ratio, both healthy and tumour-bearing rats fed the high n-3 diet had a higher ($p < 0.001$) % specific lysis compared with rats fed the low n-3 diet (Figure 5.7). In the low n-3 diet group, the tumour resulted in decreased ($p < 0.05$) NK cell cytotoxicity, while the tumour did not significantly affect NK cell cytotoxic activity when rats were fed the high n-3 diet (Figure 5.7). Lytic activity was not significantly affected by the diet or tumour (24 ± 2 lytic units, $n = 32$).

**Nitric oxide production**

**(a) High P/S Diet:** For both the low and high n-3 diet groups, nitrite ($NO_2^-$) production was not significantly different between unstimulated and Con A-stimulated splenocytes from tumour-bearing rats (**Figure 5.8**). However, for both diet groups, PMA plus Ionomycin-stimulated splenocytes produced more ($p < 0.0001$) $NO_2^-$ compared with unstimulated cells (Figure 5.8, statistics not illustrated). Feeding tumour-bearing rats long-chain n-3 fatty acids in the high P/S diet did not significantly affect $NO_2^-$ production by unstimulated splenocytes (7.2 ± 0.5 nmol/mL per $10^6$ cells) or cells stimulated with Con A (7.2 ± 0.4 nmol/mL per $10^6$ cells) or PMA plus Ionomycin (9.8 ± 0.5 nmol/mL per $10^6$ cells) ($n = 14$) (Figure 5.8).

**(b) Low P/S Diet:** $NO_2^-$ production from Con A-stimulated splenocytes was higher ($p < 0.03$) than that from unstimulated cells in the healthy, low n-3-fed group, but not the other three groups (**Figure 5.9**, statistics not illustrated). For all groups, $NO_2^-$ production was higher ($p < 0.05$) from splenocytes stimulated with PMA plus Ionomycin compared with unstimulated cells (Figure 5.9, statistics not illustrated). Both healthy and tumour-bearing rats fed long-chain n-3 fatty acids had increased ($p < 0.04$) $NO_2^-$ production by unstimulated and PMA plus Ionomycin-stimulated splenocytes (Figure 5.9). $NO_2^-$ production by Con A-stimulated splenocytes was also higher for both healthy and tumour-bearing rats fed long-chain n-3 fatty acids, but this did not reach statistical significance (Figure 5.9). In both the low and high n-3 diet groups, unstimulated and PMA plus Ionomycin-stimulated splenocytes from tumour-bearing rats produced higher ($p \leq 0.01$) levels of $NO_2^-$ compared with splenocytes from healthy rats (Figure 5.9). As well, Con A-stimulated splenocytes from tumour-bearing rats produced higher levels of $NO_2^-$ compared with splenocytes from healthy rats, but this did not reach statistical significance (Figure 5.9).

**IL-2 production**

For all groups, splenocytes cultured for 48 h without mitogen (unstimulated) produced undetectable levels of IL-2 in the culture supernatants (results not shown).

**(a) High P/S Diet:** Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect IL-2 production by splenocytes from tumour-bearing rats stimulated for 48 h with either Con A (1468 ± 75 pg/mL per $10^6$ cells) or PMA plus Ionomycin (3853 ± 195 pg/mL per $10^6$ cells) ($n = 13$) (**Figure 5.10**).

**(b) Low P/S Diet:** There was a significant interaction between long-chain n-3 fatty acids and the tumour on splenocyte IL-2 production after Con A stimulation (**Figure 5.11**). For healthy rats, splenocytes from high n-3-fed rats produced higher levels of IL-2 when stimulated with Con A or PMA plus Ionomycin, compared with splenocytes from low n-

3-fed rats (Figure 5.11). Diet did not significantly affect mitogen-stimulated IL-2 production in tumour-bearing rats (Figure 5.11).

**Fatty acid composition of R3230AC tumour cell phospholipids.** Dietary long-chain n-3 fatty acids and P/S ratio significantly altered the fatty acid composition of PC, PE, and PI of R3230AC tumour cells (**Tables 5.7.-5.9 and Figures 5.12-5.14**).

**(a) Effect of dietary long-chain n-3 fatty acids.** For both the high and low P/S diets, tumour cells from rats fed long-chain n-3 fatty acids had a significantly higher total n-3 fatty acid content and a significantly lower n-6/n-3 ratio in PC, PE and PI (Figures 5.12-5.13). Specifically, in PC, PE and PI the % of C20:5(3), C22:5(3) and C22:6(3) were increased (Figure 5.14) while the % of C22:4(6) and C22:5(6) were decreased (Tables 5.7-5.9) when rats were fed long-chain n-3 fatty acids in either the high or the low P/S diets. For both P/S diets, tumour cells from rats fed long-chain n-3 fatty acids had a significantly decreased % of C20:4(6) in PC and PE (Tables 5.7-5.8), but not PI (Table 5.9). However, the total n-6 fatty acid content in PC, PE and PI was significantly decreased in the high n-3 diet group (Tables 5.7-5.9). There was no effect of dietary long-chain n-3 fatty acids on the % of C18:2(6), total saturated or monounsaturated fat content or unsaturation index (total number of double bonds) in PC, PE and PI (Tables 5.7-5.9). Dietary long-chain n-3 fatty acids significantly decreased the P/S ratio in PC (Table 5.7).

**(b) Effect of dietary P/S ratio.** Long-chain n-3 fatty acid incorporation into tumour cell PC, PE and PI was further affected by the P/S ratio of the diet (Tables 5.7-5.9, Figures 5.12-5.14). Specifically, when rats were fed long-chain n-3 fatty acids in the low P/S diet, total n-3 content was significantly increased in PC, PE and PI (Figure 5.12) and the n-6/n-3 ratio was significantly decreased in PE and PI (Figure 5.13) compared with long-chain n-3 fatty acids fed in the high P/S diet. Specifically, the % of C20:5(3), C22:5(3), C22:6(3) were increased in PC, PE and PI when rats were fed long-chain n-3 fatty acids in the low P/S diet compared with the high P/S diet (Figure 5.14). In contrast, dietary P/S ratio did not affect total n-3 content or the % of C20:5(3), C22:5(3), C22:6(3) in PC, PE and PI when rats were fed the low n-3 diet (Figures 5.12 and 5.14), although the n-6/n-3 ratio was significantly decreased in PE and PI when rats were fed the low P/S diet (Figure 5.13). Long-chain n-3 fatty acids fed in the low P/S diet resulted in an increased P/S ratio and unsaturation index in tumour cell PE (Table 5.8). In PC the % of C18:2(6) was significantly increased when rats were fed the low P/S diet compared with the high P/S diet (Table 5.7). The % of C20:4(6) and C22:4(6) in PC, PE and PI were not affected by dietary P/S ratio (Tables 5.7-5.9). However, the % of C22:5(6) was significantly decreased in PC and PE when rats were the low P/S diet compared with the high P/S diet (Tables 5.7-5.8). There was no effect of dietary P/S ratio on total saturated, monounsaturated or n-6 fatty acid content in PC, PE and PI (Tables 5.7-5.9).

## 5.4 DISCUSSION

### Diet composition and tumour growth

In the present study, feeding long-chain n-3 fatty acids (5% w/w of total fat) in a high P/S diet did not inhibit R3230AC mammary tumour growth (Figure 5.3). When rats were fed the same level of long-chain n-3 fatty acids in a low P/S diet, there was a trend towards suppression (-31%) of tumour growth, but this effect did not reach statistical significance (Figure 5.3). Our experimental diets contained ~ 40% of energy from fat from a mixture of sources and were designed to reflect the level and composition of fat in the North American diet (Field et al. 1985) with n-6/n-3 fatty acid ratios in the long-chain n-3 fatty acid-supplemented diets of 7.8 and 3.8 in the high and low P/S diets, respectively. Other studies have reported that n-6/n-3 fatty acid ratios from 2 to 0.5 are optimal for anticancer benefits (Calviello et al. 1998, Cohen et al. 1993, Abou-El-Ela et al. 1989, Karmali 1987). However, since it is difficult to achieve an n-6/n-3 ratio less than 2 in the North American diet, the physiological relevance of such dietary designs for cancer prevention studies is not clear. In the present study, the absolute level of long-chain n-3 fatty acids was considerably lower (and the corresponding n-6/n-3 fatty acid ratios were much higher) than many previous animal studies which have shown a tumour inhibitory effect of dietary EPA and DHA (Calviello et al. 1998, Cohen et al. 1993, Abou-El-Ela et al. 1989, Karmali 1987). Although we did not find a significant effect of diet on R3220AC tumour growth at 17 d post-implantation, this does not preclude benefits of these fatty acids on later cancer stages, such as improved response to chemotherapy (de Salis and Meckling-Gill 1995), reduction of tumour metastasis (Rose et al. 1995), or prevention of cancer cachexia (Barber et al. 1999), or on earlier stages, such as cancer prevention (Noguchi et al. 1997). For example, Noguchi et al. (1997) showed that low-dose EPA and DHA inhibit the incidence of rat mammary tumours, but not their growth.

### Diet and tumour cell membrane composition

Our study confirms previous findings that changing dietary fat composition significantly alters tumour cell membrane composition (Calviello et al. 1998, Noguchi et al. 1997, Rose et al. 1995, Jenski et al. 1993, Karmali et al. 1984). However, the effect of dietary fatty acids on individual membrane phospholipid fractions in tumour cells has not been extensively studied. Such a separation is important because different phospholipid fractions may respond to dietary fat manipulation to varying degrees (Field et al. 1990). As expected, tumour cells from rats fed long-chain n-3 fatty acids had a higher total n-3 content and a lower n-6/n-3 ratio in PC, PE, and PI (Figures 5.12-5.13). Furthermore, the magnitude of increase in incorporation of long-chain n-3 fatty acids, especially EPA (C20:5n-3), docosapentaenoic acid (C22:5n-3, DPA), and DHA (C22:6n-3) into tumour phospholipids was significantly greater when long-chain n-3 fatty acids were fed in a low P/S diet (Figure 5.14). Although feeding long-chain n-3 fatty acids did not affect the amount of linoleic acid (C18:2n-6, LA) in tumour cell phospholipids, the amount of arachidonic acid (C20:4n-6, AA) was decreased in PC and PE when rats were fed EPA and DHA (Tables 5.7-5.8). Rose et al. (1995) also demonstrated that the accumulation of n-3 fatty acids in human breast cancer cell phospholipids was at the expense of AA, but not LA. It is not known whether dietary fat-induced alterations in tumour cell lipid composition are directly associated with changes in eicosanoid production, receptor or enzyme function, cell permeability, or second messenger pathways involved in tumour growth in this model. Dietary fat modulation of eicosanoids (Karmali 1987) and intracellular second messengers has been reported in other studies (Chapkin et al. 1997). Additionally, incorporation of dietary n-3 fatty acids into tumour cell membranes is associated with increased susceptibility of tumours to cell-mediated lysis (Jenski et al.

1993) and increased sensitivity to chemotherapy (de Salis and Meckling-Gill 1995). The length of time that diets were fed in the present study (17 d post-implantation) was sufficient to alter tumour cell phospholipid composition, but perhaps not long enough for these mechanisms to impact on tumour growth.

### R3230AC tumour and host immune function

Components of both the innate (natural) and acquired (specific) immune systems are important contributors to host defense against tumour cells (Whiteside and Herberman 1995, Robins 1986,). There are many putative mechanisms of immune cell cytotoxicity, including production, by CD4$^+$ Th1 cells, of IL-2 which activates NK cells (Whiteside and Herberman 1995). Nitric oxide, produced by various activated immune cells, has also been identified as a key molecule in anticancer defense and a potential mediator of NK cell cytotoxicity (Cifone et al. 1995). Although there is limited data on the effect of the R3230AC mammary tumour on host immune function, previous work has shown that anticancer immunity declines progressively with growth of other transplantable tumours, such as the Morris Hepatoma 7777 (Shewchuk et al. 1996).

In the present study, the effect of the R3230AC tumour on host immunity was evaluated in the low P/S diet experiment which included both healthy and tumour-bearing rats. At 17 d post-implantation, tumour burden had a suppressive effect on NK cell cytotoxicity, but only when rats were fed the low n-3 diet without long-chain n-3 fatty acids (Figure 5.7). In addition, dietary long-chain n-3 fatty acids increased NK cell cytotoxicity in both healthy and tumour-bearing rats, suggesting that feeding the high n-3 diet may prevent tumour-induced suppression of NK cell cytotoxicity. Healthy rats fed long-chain n-3 fatty acids also had higher splenocyte nitric oxide and IL-2 production (Figures 5.9 and 5.11, respectively). Both of these immune mediators have been proposed to play roles in the tumouricidal capacity of NK cells (Cifone et al. 1995, Ortaldo et al. 1984), but their precise role in our model requires further work. However, the increased cytotoxicity of NK cells from tumour-bearing rats fed the high n-3 diet was associated with elevated nitric oxide, but not IL-2 production (Figures 5.9 and 5.11, respectively).

Not all immune parameters assayed were suppressed in tumour-bearing animals. For example, compared with healthy rats, tumour-bearing rats had a higher proportion of $CD4^+$ T helper cells, which can produce IL-2 and other cytokines (Whiteside and Herberman 1995), $CD8^+$ T suppressor/cytotoxic cells, which can generate a population of cytotoxic T-lymphocytes (Robins 1986), and cells expressing CD28, an important co-stimulatory signal for T cell proliferation, cytokine production and cytokine receptor expression (Blair et al. 1997). In addition, tumour-bearing rats had a higher proportion of $CD4^+$ T helper cells and $CD28^+$ cells that were activated after mitogen stimulation, as determined by expression of the IL-2 receptor (CD25) (Table 5.6). Overall, tumour-bearing animals had increased CD25 expression on T cells after Con A stimulation, suggesting that cells from tumour-bearing rats may be better able to respond to an immune challenge. Tumour-bearing rats also had a lower proportion of activated $CD25^+$ B cells in spleen (Table 5.6), which may be due to the migration of these effector cells to the tumour site for initiation of tumour cell lysis (Fujiwara and Hamaoka 1995). Overall, our results have shown that various assays of antitumour immunity need to be performed in order to thoroughly evaluate host immune status in rats bearing the R3230AC tumour.

### Diet and host immune function

A major focus of current research in immunology and oncology is the development of methods to augment host antitumour immune defense. Feeding healthy rats long-chain n-3 fatty acids in a high P/S diet upregulated the host immune system (Chapter 3). However, in contrast to previous findings using healthy rats (Chapter 3), long-chain n-3 fatty acids fed in a high P/S diet did not significantly alter immune function in tumour-bearing rats. It is not known why immune responses to long-chain n-3 fatty acids fed in a high P/S diet differed between healthy and tumour-bearing rats. Our results suggest that the presence of a tumour interferes with the ability of long-chain n-3 fatty acids to enhance immune function when fed in a high P/S diet. The immunoenhancing effect of long-chain n-3 fatty acids may also be associated with their dietary level in relation to the overall polyunsaturated fat content of the diet, or the n-6/n-3 ratio, just as n-3 fatty acids are more effective at inhibiting tumour growth when the proportion of n-3 fatty acids is much greater than that of n-6 fatty acids (Ip 1997).

Interestingly, we found that long-chain n-3 fatty acids improved certain immune defenses in both healthy and tumour-bearing rats when fed in a low P/S diet with a 2-fold lower n-6/n-3 ratio compared with the high P/S diet. For example, rats fed long-chain n-3 fatty acids in a low P/S diet had significantly increased NK cell cytotoxicity (Figure 5.7), a higher proportion of $CD8^+$ and $CD28^+$ cells that were activated ($CD25^+$) (Table 5.6), and increased nitric oxide and IL-2 production after mitogen stimulation (Figures 5.9 and 5.11, respectively), whereas these immune enhancements were not found when EPA and DHA were supplemented in a high P/S diet. As discussed in Chapter 3, our results contradict many previous studies which have concluded that consumption of large, unphysiological (with respect to the human diet) amounts of long-chain n-3 fatty acids is immunosuppressive (Calder 1998). However, a recent study by Sasaki et al. (1999) also found that diets rich in DHA exert some of their immunomodulatory effects by an upregulation of the CD28-mediated costimulatory signal.

We have shown that long-chain n-3 fatty acids improve aspects of immunity in healthy rats when supplemented in either a high P/S diet (Chapters 3 and 4) or a low P/S diet, as seen in the present study. Interestingly, the same diet effect is not observed in tumour-bearing animals since our results demonstrate an immunoenhancing effect of dietary long-chain n-3 fatty acids in tumour-bearing rats when fed in a low P/S, but not high P/S, diet. Dietary long-chain n-3 fatty acids fed in a low P/S diet had further enhancing effects when fed to tumour-bearing rats as evidenced by an increase in activated $CD25^+$ T cells after mitogen stimulation compared with those fed the low n-3 diet (Table 5.6). Since tumour growth was not significantly affected by long-chain n-3 fatty acid supplementation (Figure 5.3), the potential importance of diet-associated changes in the measured immune variables is not clear. However, the ability of dietary EPA and DHA to enhance the immune system should not be overlooked, since improved host immunity may be beneficial in respects other than primary tumour growth inhibition, such as better response to chemotherapy, prevention of disease recurrence and metastasis, or infectious complications.

## Conclusions

The present study demonstrated that the polyunsaturated fat content of the diet significantly influences the effect of dietary long-chain n-3 fatty acids on immune parameters assayed in tumour-bearing animals. Long-chain n-3 fatty acids fed in the high P/S diet did not affect tumour growth or host immune responses. In contrast, feeding long-chain n-3 fatty acids in a low P/S diet enhanced antitumour immune defense in both healthy and tumour-bearing rats, increased n-3 fatty acid incorporation into tumour cell phospholipids and showed a non-significant trend towards reducing tumour growth. Thus, although long-chain n-3 fatty acids fed at physiological levels did not inhibit R3230AC tumour growth, our data suggest that they do enhance immune parameters involved in antitumour defense when fed in a low P/S diet (P/S = 0.35) Current recommendations for the North American population are to increase consumption of polyunsaturated fat (with a decrease in saturated fat intake) to achieve a P/S ratio of 1 (American Diabetes Association 1999, Krauss et al. 1996, Health and Welfare Canada 1990), which is the same ratio fed in the high P/S diet in the present study. Our results, in an animal model of breast cancer, suggest that a lower level of polyunsaturated fat than is currently recommended by several health agencies may be required to enhance the immune benefits of long-chain n-3 fatty acids, particularly during cancer.

TABLE 5.1 Fatty Acid Composition of Experimental Diets Fed to Rats[1]

| | High P/S Diet | | Low P/S Diet | |
|---|---|---|---|---|
| | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet |
| **Fatty Acid** | % w/w | | | |
| C14:0 | 1.3 | 2.0 | 2.2 | 2.8 |
| C16:0 | 15.7 | 15.7 | 21.1 | 21.0 |
| C18:0 | 26.9 | 25.5 | 44.4 | 42.6 |
| C18:1 (9) | 8.4 | 8.2 | 4.6 | 4.5 |
| C18:2 (6) | 44.3 | 39.3 | 23.4 | 18.8 |
| C18:3 (3) | 1.2 | 0.2 | 1.1 | 0.2 |
| C20:5 (3) | nd | 3.2 | nd | 3.2 |
| C22:5 (3) | nd | 0.2 | nd | 0.2 |
| C22:6 (3) | nd | 0.8 | nd | 0.8 |
| SFA | 45.6 | 44.8 | 70.2 | 69.0 |
| MUFA | 8.7 | 10.4 | 4.8 | 6.6 |
| PUFA | 45.3 | 44.5 | 24.5 | 24.0 |
| n-6 PUFA | 44.3 | 39.6 | 23.4 | 19.1 |
| n-3 PUFA | 1.2 | 5.1 | 1.1 | 5.0 |
| n-6/n-3 ratio | 37 | 7.8 | 21 | 3.8 |
| P/S ratio | 1.0 | 1.0 | 0.35 | 0.35 |

[1] Values are g/100 g of total fat, except for ratios. Diets contained 200 g/kg of fat from a mixture of sources [safflower oil, hard beef tallow, linseed oil (low n-3 diets only), and fish oil (high n-3 diets only]. Minor fatty acids are not reported, therefore totals do not add up to 100%. Abbreviations used: nd, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids.

**TABLE 5.2 Monoclonal Antibodies Used in Indirect Immunofluorescence (Phenotype) Assay**

| Clone[1] | Specificity | Description |
|---|---|---|
| OX19 | CD5 | Reacts with 69 kDa surface glycoprotein on thymocytes and peripheral T cells |
| w3/25 | CD4 | Reacts with 48-53 kDa surface glycoprotein on T helper cells and peritoneal macrophages |
| OX8 | CD8a | Reacts with 34 kDa α chain of CD8 antigen on thymocytes, T suppressor/cytotoxic cells, natural killer (NK) cells, and some activated T helper cells |
| OX12 | Ig κ chain | Reacts with immunoglobulin (Ig) κ chain on B cells |
| OX42 | CD11b/c | Reacts with 160, 103, and 95 kDa cell surface proteins on most resident peritoneal and activated macrophages, granulocytes, monocytes, and dendritic cells |
| 3.2.3 | CD161 | Reacts with NKR-P1A on NK cells and subset of T cells |
| JJ319 | CD28 | Reacts with a costimulatory receptor for activation on T cells and a subset of NK cells |
| OX39 | CD25 | Reacts with the α-chain of the interleukin-2 receptor on activated T and B cells |

[1] All monoclonal antibodies were kindly provided by Dr. A. Rabinovitch (University of Alberta, Edmonton, AB, Canada) with the exceptions of 3.2.3 and OX39 (Cedarlane Laboratories Ltd., Hornby, ON, Canada) and JJ319 (PharMingen, Mississauga, ON, Canada). All mAb were mouse anti-rat IgG.

**TABLE 5.3 Effect of Diet on Food Intake, Body Weight and Spleen Weight of Rats**[1]

| | High P/S Diet | | Low P/S Diet | | | |
|---|---|---|---|---|---|---|
| | Tumour-bearing | | Healthy | | Tumour-bearing | |
| | Low n-3 ($n = 7$) | High n-3 ($n = 7$) | Low n-3 ($n = 9$) | High n-3 ($n = 10$) | Low n-3 ($n = 7$) | High n-3 ($n = 7$) |
| Food Intake, g/kg body weight/d | 58 ± 1 | 56 ± 1 | 61 ± 2 | 69 ± 5 | 64 ± 4 | 64 ± 2 |
| Final body weight, g[2] | 152 ± 4 | 156 ± 5 | 160 ± 2 | 158 ± 3 | 152 ± 2 | 161 ± 4 |
| Weight increase, g | 15 ± 4 | 18 ± 3 | 11 ± 2 | 16 ± 3 | 11 ± 3 | 12 ± 3 |
| Spleen weight, g/ kg body weight | 3.2 ± 0.1 | 3.3 ± 0.1 | 2.7 ± 0.1 | 2.9 ± 0.1 | 2.8 ± 0.1 | 2.9 ± 0.1 |
| Spleen cells (× $10^6$)/g spleen | 425 ± 32 | 378 ± 44 | 437 ± 27 | 423 ± 25 | 442 ± 32 | 433 ± 13 |

[1] Fischer 344 rats were fed one of four semi-purified diets (20% w/w fat) for 21 d pre- and 17 d post- R3230AC mammary tumour implantation (healthy rats were not implanted with a tumour). The polyunsaturated to saturated fatty acid (P/S) ratio was either 1 (high P/S diet) or 0.35 (low P/S diet). At each P/S ratio, diets were either unsupplemented (low n-3 diet) or supplemented with long-chain n-3 fatty acids (high n-3 diet). The low and high n-3 diets provided long-chain n-3 fatty acids at 0 or 5% w/w of total fat, respectively. Values are means ± SEM. *n* = number of rats per group. No significant differences were found.

[2] Final body weight includes tumour weight.

TABLE 5.4 Immune Cell Phenotypes in Freshly Isolated Splenocytes From Tumour-Bearing Rats Fed a High P/S Diet Either Unsupplemented or Supplemented With Long-Chain n-3 Fatty Acids[1]

| Immune Cell Phenotype | Low n-3 Diet | High n-3 Diet | Significance, $p \leq$ [2] |
|---|---|---|---|
| | % of total immune cells in spleen | | |
| $CD5^+$ T cells | 51 ± 2 | 51 ± 1 | NS |
| $CD4^+$ T helper cells | 31 ± 1 | 30 ± 1 | NS |
| $CD8^+$ T suppressor/cytotoxic cells | 17 ± 2 | 19 ± 1 | NS |
| B cells | 31 ± 2 | 30 ± 2 | NS |
| Macrophages | 3.9 ± 0.7 | 4.1 ± 0.5 | NS |
| Natural killer cells | 4.1 ± 0.4 | 2.6 ± 0.2 | 0.01 |
| CD4/CD8 | 2.0 ± 0.2 | 1.6 ± 0.1 | NS |
| $CD25^+$ cells | 0.71 ± 0.07 | 0.67 ± 0.09 | NS |

[1] Immune cell phenotypes were identified by indirect immunofluorescence assay and flow cytometry as described in Materials and Methods. Values are means ± SEM (*n* = 7/group).

[2] Significant effect of diet as determined by a Student's *t*-test. NS = $p > 0.05$.

**TABLE 5.5 Immune Cell Phenotypes in Freshly Isolated Splenocytes From Healthy and Tumour-Bearing Rats Fed a Low P/S Diet Either Unsupplemented or Supplemented With Long-Chain n-3 Fatty Acids[1]**

| | Healthy | | Tumour-bearing | | Significance, p ≤ [2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenotype** | **Low n-3** | **High n-3 Diet** | **Low n-3 Diet** | **High n-3 Diet** | Diet | Tumour |
| | % of total immune cells in spleen | | | | | |
| CD5$^+$ T cells | 53 ± 1 | 50 ± 1 | 54 ± 1 | 49 ± 1 | 0.0003 | NS |
| CD4$^+$ T helper cells | 31 ± 1 | 28 ± 1 | 32 ± 2 | 26 ± 2 | 0.005 | NS |
| CD8$^+$ T suppressor/cytotoxic cells | 13 ± 1 | 14 ± 1 | 18 ± 2 | 15 ± 2 | NS | 0.04 |
| B cells | 26 ± 1 | 28 ± 1 | 25 ± 1 | 25 ± 1 | NS | 0.03 |
| Macrophages | 2.9 ± 0.4 | 3.6 ± 0.5 | 3.4 ± 0.4 | 2.6 ± 0.5 | NS | NS |
| Natural killer cells | 5.3 ± 0.7 | 5.0 ± 0.5 | 4.0 ± 0.5 | 4.6 ± 0.8 | NS | NS |
| CD4/CD8 | 2.3 ± 0.2 | 2.1 ± 0.2 | 1.9 ± 0.2 | 2.0 ± 0.4 | NS | NS |
| CD28$^+$ cells | 0.79 ± 0.13 | 0.73 ± 0.06 | 0.83 ± 0.04 | 0.70 ± 0.03 | NS | NS |
| CD25$^+$ cells | 1.0 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 | 1.1 ± 0.1 | NS | NS |

[1] Immune cell phenotypes were identified by indirect immunofluorescence assay and flow cytometry as described in Materials and Methods. Values are means ± SEM ($n \geq 6$/group).

[2] Significant effect of diet and tumour as determined by two-way ANOVA, NS = $p > 0.05$. No significant interactions were found.

**TABLE 5.6 Interleukin-2 Receptor (CD25) Expression in Splenocytes From Healthy and Tumour-Bearing Rats Fed a Low P/S Diet After 48 h Splenocyte Stimulation with Concanavalin A**[1]

| | Healthy | | Tumour-bearing | | Significance, $p \leq$ [2] | |
|---|---|---|---|---|---|---|
| Immune Cell Phenotype | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Tumour |
| | % of total immune cells in spleen | | | | | |
| $CD25^+ CD5^+$ T cells | $25 \pm 2^b$ | $26 \pm 2^b$ | $30 \pm 3^b$ | $43 \pm 4^a$ | 0.02 | 0.0004 |
| $CD25^+ CD4^+$ T helper cells | 19 ± 2 | 21 ± 2 | 23 ± 2 | 29 ± 3 | NS | 0.01 |
| $CD25^+ CD8^+$ T suppressor/cytotoxic cells | 10 ± 1 | 12 ± 1 | 11 ± 1 | 15 ± 2 | 0.03 | NS |
| $CD25^+$ B cells | 34 ± 4 | 33 ± 3 | 22 ± 1 | 18 ± 2 | NS | 0.0003 |
| $CD25^+$ Macrophages | 6.7 ± 0.4 | 6.2 ± 0.7 | 5.8 ± 0.7 | 4.5 ± 0.4 | NS | 0.04 |
| $CD25^+ CD28^+$ cells | 19 ± 1 | 24 ± 2 | 30 ± 3 | 42 ± 3 | 0.03 | 0.0008 |

[1] Immune cell phenotypes were identified by indirect immunofluorescence assay and flow cytometry as described in Materials and Methods. Values are means ± SEM ($n \geq 5$/group).

[2] Significant effect of diet and tumour as determined by two-way ANOVA, NS = $p > 0.05$. When a significant interaction was found, means within a row without a common superscript are statistically different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

**TABLE 5.7 Fatty Acid Composition of Phosphatidylcholine of R3230AC Mammary Tumour Cells Isolated From Rats Fed Different Diets[1]**

| | High P/S Diet (P/S = 1) | | Low P/S Diet (P/S = 0.35) | | Significance, p ≤ [2] | |
|---|---|---|---|---|---|---|
| | Low n-3 | High n-3 | Low n-3 | High n-3 | n-3 | P/S |
| Fatty Acid | % w/w of total fatty acids | | | | | |
| C14:0 | 0.98 ± 0.18 | 1.2 ± 0.2 | 1.1 ± 0.2 | 1.3 ± 0.2 | NS | NS |
| C15:0 | 0.24 ± 0.02 | 0.26 ± 0.06 | 0.14 ± 0.05 | 0.11 ± 0.05 | NS | 0.02 |
| C16:0 | 27 ± 1 | 29 ± 1 | 28 ± 1 | 28 ± 1 | NS | NS |
| C16:1 (7) | 2.9 ± 0.2 | 3.1 ± 0.2 | 2.7 ± 0.2 | 2.4 ± 0.2 | NS | 0.04 |
| C16:1 (5) | 0.96 ± 0.06 | 1.1 ± 0.1 | 0.94 ± 0.04 | 1.1 ± 0.1 | 0.04 | NS |
| C17:0 | 0.52 ± 0.07 | 0.64 ± 0.17 | 0.38 ± 0.07 | 0.36 ± 0.06 | NS | NS |
| C18:0 | 14 ± 1 | 14 ± 1 | 14 ± 1 | 14 ± 1 | NS | NS |
| C18:1 (9) | 16 ± 1 | 17 ± 1 | 16 ± 1 | 17 ± 1 | NS | NS |
| C18:1 (7) | 6.3 ± 0.2 | 6.2 ± 0.2 | 6.3 ± 0.2 | 7.0 ± 0.2 | NS | NS |
| C18:2 (6) | 3.6 ± 0.2 | 4.0 ± 0.4 | 4.7 ± 0.2 | 5.0 ± 0.4 | NS | 0.01 |
| C18:3 (6) | 0.17 ± 0.03 | 0.11 ± 0.04 | 0.12 ± 0.02 | 0.10 ± 0.02 | NS | NS |
| C18:3 (3) | 0.16 ± 0.06 | 0.25 ± 0.18 | nd | nd | NS | NS |
| C20:1 (9) | 0.27 ± 0.04 | 0.30 ± 0.08 | 0.22 ± 0.03 | 0.21 ± 0.02 | NS | NS |
| C20:1 (7) | 0.33 ± 0.02 | 0.29 ± 0.02 | 0.44 ± 0.09 | 0.32 ± 0.03 | NS | NS |
| C20:2 (6) | 0.46 ± 0.10 | 0.25 ± 0.12 | 0.20 ± 0.07 | 0.31 ± 0.07 | NS | NS |
| C20:3 (9) | 1.1 ± 0.1 | 0.86 ± 0.03 | 0.82 ± 0.14 | 1.1 ± 0.02 | NS | NS |
| C20:3 (6) | 0.52 ± 0.05$^{a}$ | 0.43 ± 0.03$^{a}$ | 0.31 ± 0.01$^{b}$ | 0.40 ± 0.02$^{ab}$ | NS | 0.005 |
| C20:4 (6) | 15 ± 0.4 | 12 ± 1 | 15 ± 0.3 | 12 ± 0.1 | 0.0001 | NS |
| C22:0 | 0.19 ± 0.03 | 0.23 ± 0.06 | 0.17 ± 0.02 | 0.17 ± 0.02 | NS | NS |
| C22:1 (9) | 0.10 ± 0.02 | nd | 0.13 ± 0.01 | 0.13 ± 0.01 | NS | 0.04 |
| C22:1 (7) | 0.30 ± 0.08 | 0.49 ± 0.20 | 0.17 ± 0.04 | 0.21 ± 0.04 | NS | NS |
| C22:4 (6) | 2.6 ± 0.1 | 1.4 ± 0.1 | 2.9 ± 0.2 | 1.4 ± 0.1 | 0.0001 | NS |
| C22:5 (6) | 0.78 ± 0.05 | 0.37 ± 0.02 | 0.70 ± 0.03 | 0.27 ± 0.01 | 0.0001 | 0.02 |
| SFA | 44 ± 1 | 46 ± 1 | 44 ± 1 | 44 ± 1 | NS | NS |
| MUFA | 28 ± 1 | 29 ± 1 | 27 ± 1 | 29 ± 1 | NS | NS |
| n-6 PUFA | 23 ± 1 | 20 ± 1 | 24 ± 1 | 19 ± 1 | 0.0001 | NS |
| P/S ratio | 0.61 ± 0.03 | 0.51 ± 0.01 | 0.61 ± 0.02 | 0.58 ± 0.01 | 0.01 | NS |
| U. I. | 128 ± 3 | 119 ± 3 | 128 ± 3 | 126 ± 2 | NS | NS |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM (*n* ≥ 5 per diet). Abbreviations used: nd, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; n-6 PUFA, sum of n-6 polyunsaturated fatty acids; n-3 PUFA, sum of n-3 polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids; U. I., unsaturation index (total number of double bonds).

[2] Significant effect of dietary n-3 fatty acids and P/S ratio as determined by two-way

ANOVA, NS = $p > 0.05$. When a significant interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test. Values for phosphatidylcholine C20:5(3), C22:5(3), C22:6(3), total n-3 fatty acids, and n-6/n-3 ratio are illustrated in Figures 5.12-5.14.

**TABLE 5.8 Fatty Acid Composition of Phosphatidylethanolamine of R3230AC Mammary Tumour Cells Isolated From Rats Fed Different Diets**[1]

| | High P/S Diet (P/S = 1) | | Low P/S Diet (P/S = 0.35) | | Significance, p | |
|---|---|---|---|---|---|---|
| | Low n-3 | High n-3 | Low n-3 | High n-3 | n-3 | P/S |
| **Fatty Acid** | % (w/w) of total fatty acids | | | | | |
| C14:0 | 0.23 ± 0.07 | 0.46 ± 0.13 | 0.26 ± 0.04 | 0.25 ± 0.03 | NS | NS |
| C15:0 | 0.13 ± 0.04 | 0.26 ± 0.13 | 0.12 ± 0.03 | nd | NS | NS |
| C16:0 | 7.0 ± 0.5 | 11 ± 2 | 6.1 ± 0.5 | 5.7 ± 0.4 | NS | 0.03 |
| C16:1 (7) | 0.56 ± 0.01 | 0.61 ± 0.12 | 0.48 ± 0.09 | 0.29 ± 0.14 | NS | NS |
| C16:1 (5) | 0.50 ± 0.02 | 0.36 ± 0.11 | 0.61 ± 0.04 | 0.66 ± 0.03 | NS | 0.01 |
| C17:0 | 0.48 ± 0.05 | 1.0 ± 0.3 | 0.33 ± 0.06 | 0.36 ± 0.08 | NS | NS |
| C18:0 | 18 ± 0.4 | 20 ± 1 | 17 ± 0.5 | 17 ± 0.3 | NS | 0.04 |
| C18:1 (9) | 11 ± 0.1 | 11 ± 1 | 12 ± 0.3 | 13 ± 0.3 | NS | NS |
| C18:1 (7) | $2.6 \pm 0.1^{ab}$ | $2.2 \pm 0.1^{c}$ | $2.4 \pm 0.04^{bc}$ | $2.8 \pm 0.04^{a}$ | NS | NS |
| C18:2 (6) | 1.9 ± 0.1 | 2.5 ± 0.3 | 1.7 ± 0.2 | 1.9 ± 0.1 | NS | NS |
| C20:1 (9) | 0.22 ± 0.02 | 0.28 ± 0.09 | 0.20 ± 0.02 | 0.19 ± 0.02 | NS | NS |
| C20:1 (7) | 0.15 ± 0.04 | 0.10 ± 0.05 | 0.21 ± 0.01 | 0.21 ± 0.02 | NS | 0.04 |
| C20:2 (6) | 0.42 ± 0.03 | 0.61 ± 0.13 | 0.33 ± 0.07 | 0.30 ± 0.11 | NS | NS |
| C20:3 (9) | 1.1 ± 0.05 | 0.89 ± 0.15 | 1.2 ± 0.1 | 1.3 ± 0.1 | NS | 0.02 |
| C20:3 (6) | 0.25 ± 0.03 | 0.24 ± 0.10 | 0.25 ± 0.04 | 0.32 ± 0.03 | NS | NS |
| C20:4 (6) | 29 ± 1 | 24 ± 2 | 29 ± 1 | 25 ± 1 | 0.005 | NS |
| C22:0 | 0.21 ± 0.02 | 0.25 ± 0.09 | 0.18 ± 0.03 | 0.15 ± 0.02 | NS | NS |
| C22:1 (9) | 0.06 ± 0.04 | 0.08 ± 0.03 | 0.17 ± 0.01 | 0.17 ± 0.03 | NS | 0.005 |
| C22:1 (7) | 0.45 ± 0.06 | 0.85 ± 0.26 | 0.32 ± 0.08 | 0.26 ± 0.06 | NS | 0.05 |
| C22:4 (6) | 6.4 ± 0.2 | 2.8 ± 0.1 | 6.4 ± 0.5 | 2.7 ± 0.2 | 0.0001 | NS |
| C22:5 (6) | 2.1 ± 0.1 | 1.1 ± 0.1 | 2.0 ± 0.1 | 0.67 ± 0.03 | 0.0001 | 0.003 |
| SFA | 27 ± 1 | 33 ± 4 | 25 ± 1 | 24 ± 1 | NS | NS |
| MUFA | 16 ± 1 | 16 ± 1 | 16 ± 1 | 17 ± 1 | NS | NS |
| n-6 PUFA | 40 ± 1 | 32 ± 2 | 39 ± 1 | 31 ± 1 | 0.0001 | NS |
| P/S ratio | $1.7 \pm 0.1^{ab}$ | $1.1 \pm 0.2^{b}$ | $1.8 \pm 0.1^{a}$ | $1.9 \pm 0.1^{a}$ | NS | 0.002 |
| U. I. | 202 ± 2 | 183 ± 11 | 206 ± 3 | 214 ± 5 | NS | 0.02 |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 4$ per diet). Abbreviations used are defined in the legend to Table 5.8.

[2] Significant effect of dietary n-3 fatty acids and P/S ratio as determined by two-way ANOVA, NS = $p > 0.05$. When a significant interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test. Values for phosphatidylethanolamine C20:5(3), C22:5(3), C22:6(3), total n-3 fatty acids, and n-6/n-3 ratio are illustrated in Figures 5.12-5.14.

**TABLE 5.9 Fatty Acid Composition of Phosphatidylinositol of R3230AC Mammary Tumour Cells Isolated From Rats Fed Different Diets[1]**

| | High P/S Diet (P/S = 1) | | Low P/S Diet (P/S = 0.35) | | Significance, $p \leq$ [2] | |
|---|---|---|---|---|---|---|
| | Low n-3 | High n-3 | Low n-3 | High n-3 | n-3 | P/S |
| **Fatty Acid** | % (w/w) of total fatty acids | | | | | |
| C14:0 | 0.56 ± 0.13 | 0.92 ± 0.24 | 0.54 ± 0.10 | 0.37 ± 0.13 | NS | NS |
| C15:0 | 0.20 ± 0.09$^{ab}$ | 0.48 ± 0.13$^{a}$ | 0.26 ± 0.06 | 0.12 ± 0.07 | NS | NS |
| C16:0 | 9.8 ± 1.4 | 12 ± 2 | 8.6 ± 1.2 | 7.4 ± 1.1 | NS | NS |
| C16:1 (7) | 0.61 ± 0.06 | 0.84 ± 0.15 | 0.51 ± 0.05 | 0.47 ± 0.04 | NS | 0.02 |
| C16:1 (5) | 0.14 ± 0.07 | 0.13 ± 0.05 | 0.20 ± 0.03 | 0.18 ± 0.06 | NS | NS |
| C17:0 | 0.85 ± 0.14 | 1.4 ± 0.5 | 0.73 ± 0.19 | 0.57 ± 0.13 | NS | NS |
| C18:0 | 38 ± 1 | 36 ± 1 | 38 ± 1 | 39 ± 1 | NS | NS |
| C18:1 (9) | 6.3 ± 0.1 | 7.4 ± 0.6 | 6.4 ± 0.3 | 6.7 ± 0.2 | NS | NS |
| C18:1 (7) | 2.1 ± 0.1 | 2.2 ± 0.1 | 2.2 ± 0.1 | 2.6 ± 0.1 | 0.02 | 0.04 |
| C18:2 (6) | 2.0 ± 0.2 | 2.3 ± 0.2 | 2.0 ± 0.2 | 2.0 ± 0.2 | NS | NS |
| C20:1 (9) | 0.14 ± 0.06 | 0.43 ± 0.17 | 0.38 ± 0.07 | 0.18 ± 0.07 | NS | NS |
| C20:1 (7) | nd | nd | 0.20 ± 0.03 | 0.16 ± 0.06 | NS | 0.02 |
| C20:2 (6) | 1.3 ± 0.7 | 0.56 ± 0.07 | 0.57 ± 0.04 | 0.64 ± 0.02 | NS | NS |
| C20:3 (9) | 2.6 ± 0.2 | 2.5 ± 0.3 | 3.0 ± 0.3 | 4.0 ± 0.3 | NS | 0.003 |
| C20:3 (6) | 0.36 ± 0.03$^{b}$ | 0.31 ± | 0.44 ± 0.02$^{b}$ | 0.62 ± 0.05$^{a}$ | NS | 0.002 |
| C20:4 (6) | 28 ± 2 | 27 ± 2 | 27 ± 1 | 26 ± 1 | NS | NS |
| C22:0 | 0.21 ± 0.09 | 0.12 ± 0.07 | 0.33 ± 0.05 | 0.24 ± 0.08 | NS | NS |
| C22:1 (7) | 0.90 ± 0.20 | 0.53 ± 0.18 | 0.50 ± 0.11 | 0.41 ± 0.11 | NS | NS |
| C22:4 (6) | 2.8 ± 0.3 | 1.6 ± 0.1 | 3.0 ± 0.4 | 1.8 ± 0.1 | 0.0004 | NS |
| C22:5 (6) | 0.64 ± 0.11 | 0.29 ± 0.07 | 0.65 ± 0.10 | 0.35 ± 0.03 | 0.002 | NS |
| SFA | 50 ± 1 | 51 ± 2 | 49 ± 1 | 48 ± 1 | NS | NS |
| MUFA | 10 ± 1$^{b}$ | 12 ± 1$^{a}$ | 11 ± 1$^{ab}$ | 11 ± 1$^{ab}$ | NS | NS |
| n-6 PUFA | 35 ± 1 | 31 ± 2 | 34 ± 1 | 32 ± 1 | 0.04 | NS |
| P/S ratio | 0.79 ± 0.06 | 0.70 ± 0.07 | 0.80 ± 0.04 | 0.85 ± 0.05 | NS | NS |
| U. I. | 159 ± 8 | 157 ± 6 | 162 ± 5 | 169 ± 6 | NS | NS |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 4$ per diet). Abbreviations used are defined in the legend to Table 5.8.

[2] Significant effect of dietary n-3 fatty acids and P/S ratio as determined by two-way ANOVA, NS = $p > 0.05$. When a significant interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test. Values for phosphatidylinositol C20:5(3), C22:5(3), C22:6(3), total n-3 fatty acids, and n-6/n-3 ratio are illustrated in Figures 5.12-5.14.

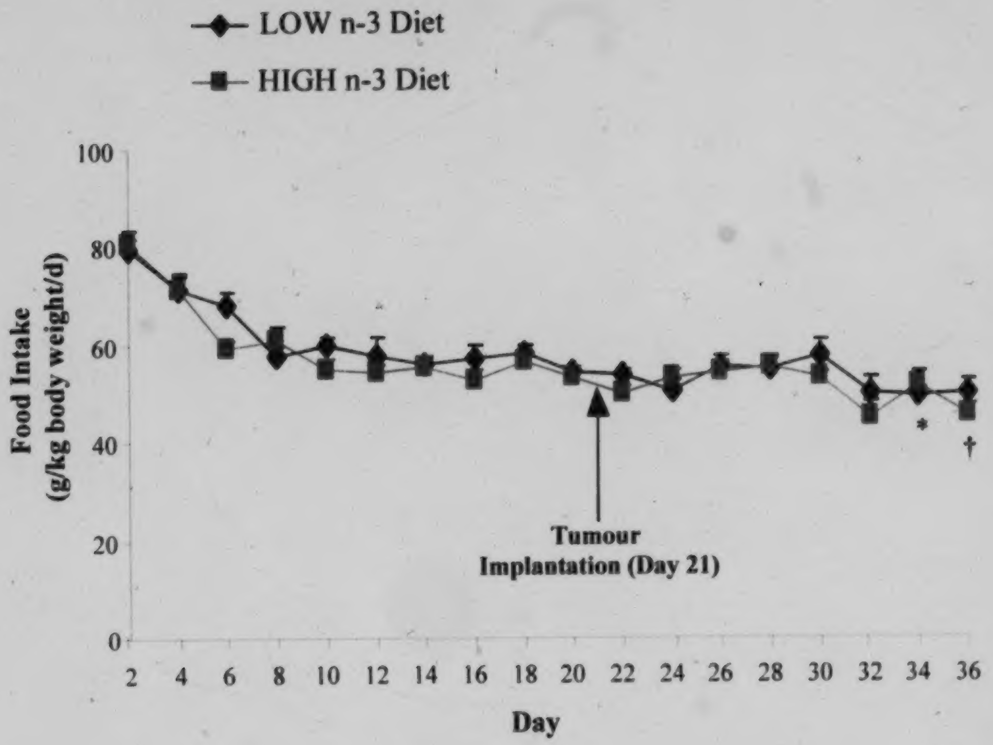

**FIGURE 5.1 Food Intake of Tumour-Bearing Rats Fed a High P/S Diet.** Rats were fed a high P/S diet (P/S = 1) either unsupplemented (low n-3 diet) or supplemented with long-chain n-3 fatty acids (high n-3 diet) for 21 d pre- and 17 d post-implantation of the R3230AC mammary tumour. The low and high n-3 diets provided long-chain n-3 fatty acids at 0 or 5% w/w of total fat, respectively. Food was weighed every 2 d and intake was calculated as g of food per kg body weight per d. Values are means ± SEM (*n* = 7/diet). Diet did not significantly affect food intake as determined by a split-plot (repeated measures) ANOVA. Compared with day 20 (prior to tumour implantation), food intake was lower on day 34 for low n-3-fed rats (* $p < 0.02$) and day 36 for high n-3-fed rats († $p < 0.02$) (by paired *t*-test with day 20).

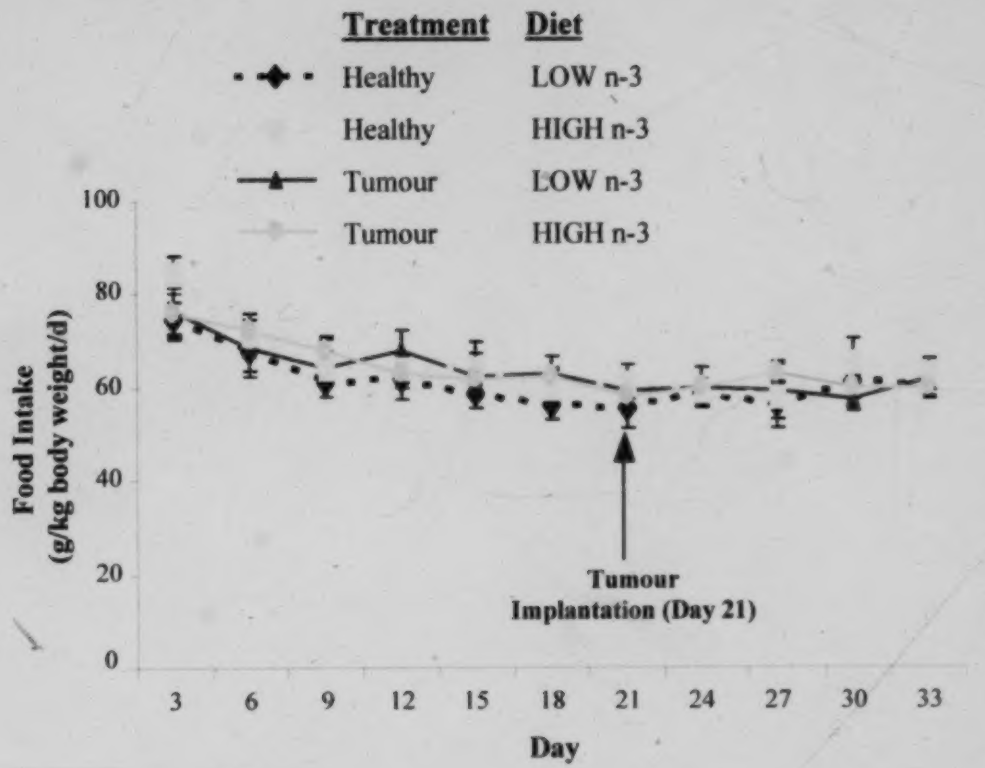

**FIGURE 5.2 Food Intake of Healthy and Tumour-Bearing Rats Fed a Low P/S Diet.** Rats were fed a low P/S diet (P/S = 0.35) either unsupplemented (low n-3 diet) or supplemented with long-chain n-3 fatty acids (high n-3 diet) for 21 d pre- and 17 d post-implantation of the R3230AC mammary tumour. The low and high n-3 diets provided long-chain n-3 fatty acids at 0 or 5% w/w of total fat, respectively. Food was weighed every 3 d and intake was calculated as g of food per kg body weight per d. Values are means ± SEM (Heathy rats, *n* ≥ 9/diet; Tumour-bearing rats, *n* = 7/diet). Neither tumour nor diet significantly affected food intake as determined by a split-plot (repeated measures) ANOVA.

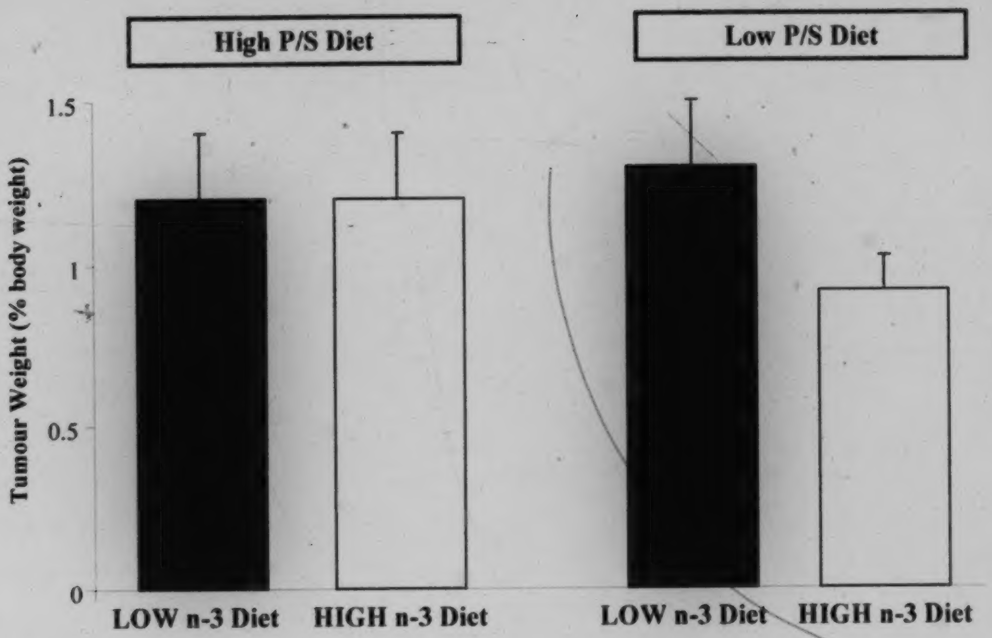

**FIGURE 5.3 Effect of Dietary Long-Chain n-3 Fatty Acids on Growth of the R3230AC Mammary Adenocarcinoma in Rats.** Fischer 344 rats were fed one of four diets (20% w/w fat) for 21 d pre- and 17 d post-implantation of the R3230AC mammary tumour. The P/S ratio was either 1 (high P/S diet) or 0.35 (low P/S diet). The low and high n-3 diets provided long-chain n-3 fatty acids at 0 or 5% w/w of total fat, respectively. Bars represent means ± SEM ($n \geq 6$/diet). Long-chain n-3 fatty acids fed in the high P/S diet did not significantly affect tumour weight. In the low P/S diet group, final tumour weight was lower (-31%) when rats were fed long-chain n-3 fatty acids, but this did not reach statistical significance (p = 0.1).

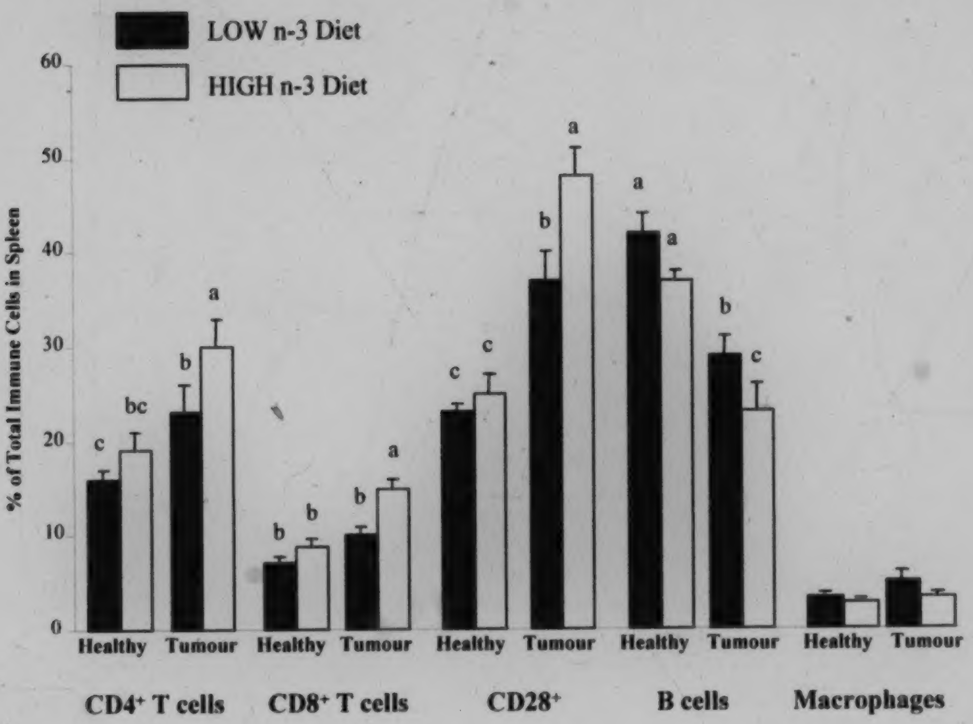

**FIGURE 5.4 Effect of Long-Chain n-3 Fatty Acids Fed in a Low P/S Diet on Immune Cell Phenotypes in Rat Spleen After 48 h Concanavalin A Stimulation.** Splenocytes were isolated from healthy or tumour-bearing rats fed a low P/S diet, with or without long-chain n-3 fatty acids. Isolated splenocytes were stimulated for 48 h with Concanavalin A (5 mg/L) and immune phenotypes were identified by indirect immunofluorescence assay and flow cytometry. Bars represent means ± SEM ($n \geq$ 7/group). The effects of diet and tumour were analyzed by two-way ANOVA. For each immune cell phenotype, bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

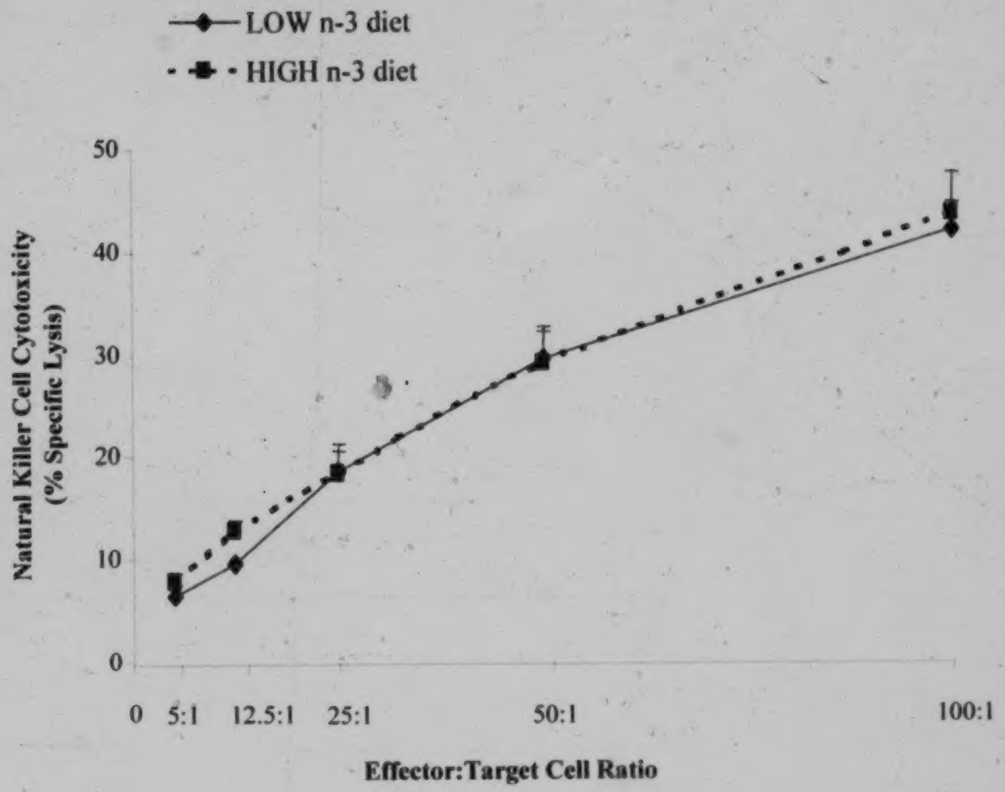

**FIGURE 5.5 Effect of Long-Chain n-3 Fatty Acids Fed in a High P/S Diet on Natural Killer Cell Cytotoxic Activity in Tumour-Bearing Rats.** Natural killer (NK) cell cytotoxic activity is expressed as % specific lysis, which is equal to 100 x (mean experimental $^{51}$Cr release from labeled YAC-1 cells – mean spontaneous $^{51}$Cr release)/(mean maximum $^{51}$Cr release – mean spontaneous $^{51}$Cr release). Points represent means ± SEM ($n \geq 5$/group). Lines were not significantly different as determined by a split-plot (repeated measures) ANOVA.

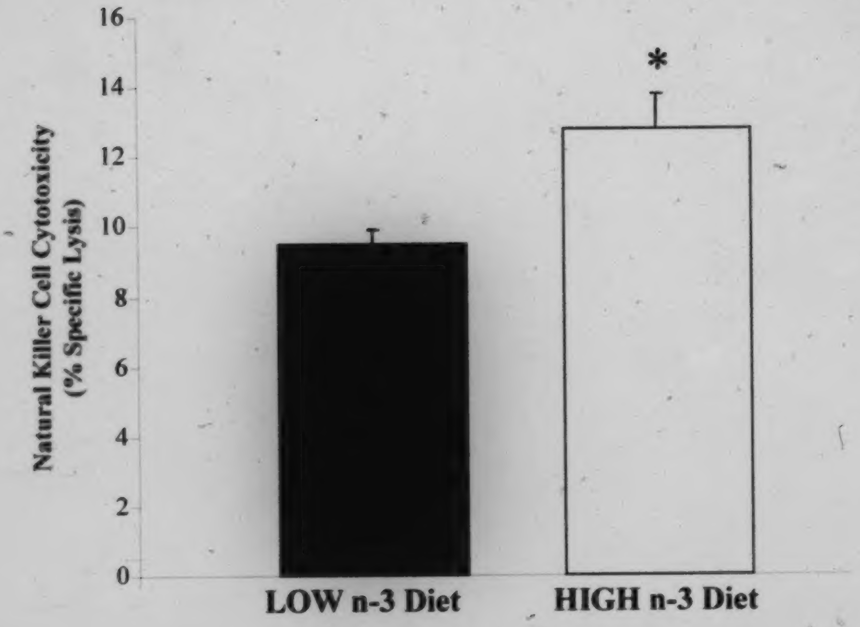

**FIGURE 5.6 Effect of Long-Chain n-3 Fatty Acids Fed in a High P/S Diet on Natural Killer Cell Cytotoxicity at the 12.5:1 Effector:Target Cell Ratio in Tumour-Bearing Rats.** Natural killer (NK) cell cytotoxic activity is expressed as % specific lysis, which is equal to 100 x (mean experimental $^{51}$Cr release from labeled YAC-1 cells – mean spontaneous $^{51}$Cr release)/(mean maximum $^{51}$Cr release – mean spontaneous $^{51}$Cr release). Bars represent means ± SEM (*n* = 6/diet). At the 12.5:1 effector:target cell ratio, the % specific lysis of target YAC-1 cells was greater for splenocytes from rats fed the high n-3 diet compared with splenocytes from those fed the low n-3 diet (* $p < 0.01$).

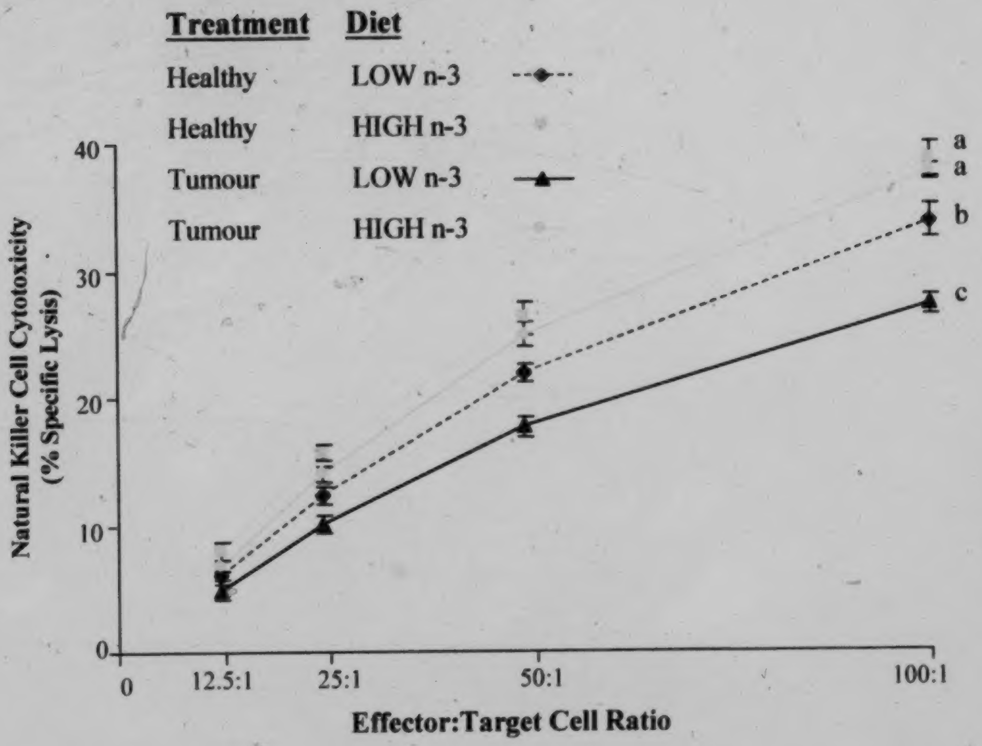

**FIGURE 5.7 Effect of Long-Chain n-3 Fatty Acids Fed in a Low P/S Diet on Natural Killer Cell Cytotoxic Activity in Healthy and Tumour-Bearing Rats.** Natural killer (NK) cell cytotoxic activity is expressed as % specific lysis, which is equal to 100 x (mean experimental $^{51}$Cr release from labeled YAC-1 cells – mean spontaneous $^{51}$Cr release)/(mean maximum $^{51}$Cr release – mean spontaneous $^{51}$Cr release). Points represent means ± SEM ($n \geq 7$/group). Lines that do not share a common letter are significantly different ($p < 0.05$) as determined by a two-way split-plot (repeated measures) ANOVA.

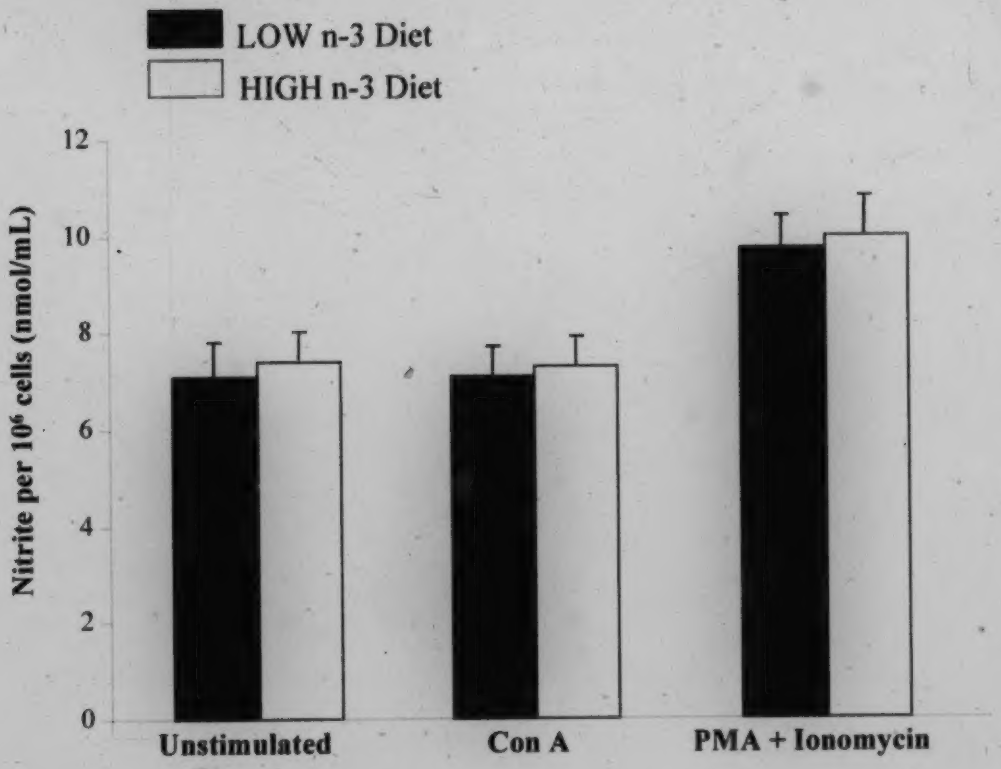

**FIGURE 5.8 Effect of Long-Chain n-3 Fatty Acids Fed in a High P/S Diet on Splenocyte Nitric Oxide Production in Tumour-Bearing Rats.** Cells were cultured without mitogen (unstimulated) or with Concanavalin A (Con A, 5 mg/L) or Phorbol Myrisate Acetate (PMA, 30 μg/L) plus Ionomycin (0.75 μmol/L) for 48 h. The concentration of nitrite ($NO_2^-$) in cell culture supernatants was determined by the Griess reaction as described in Materials and Methods. Bars represent means ± SEM (*n* = 7/group). No significant differences were found.

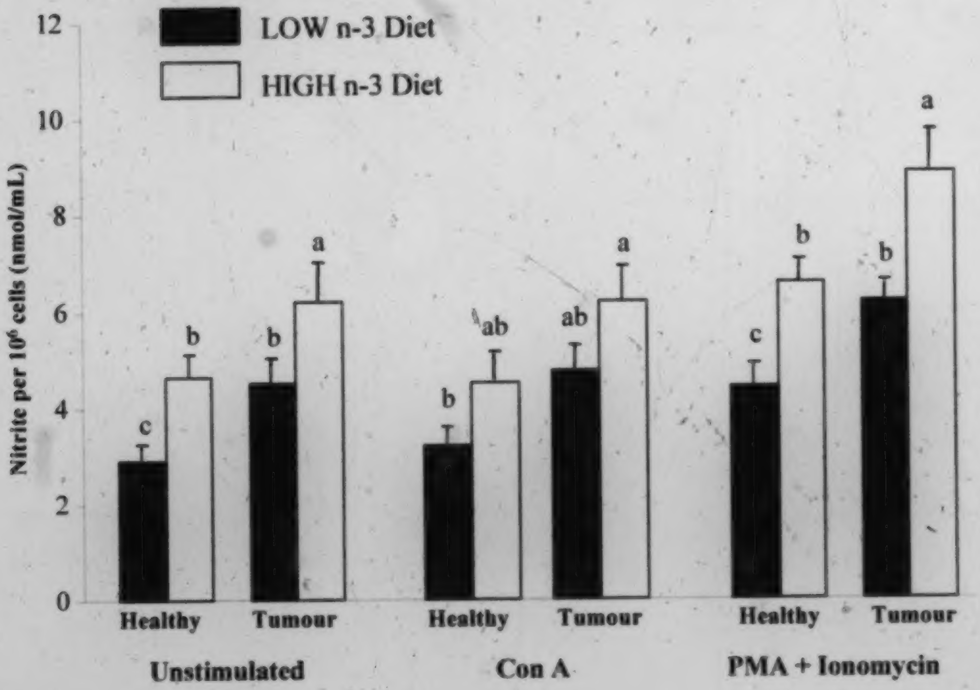

**FIGURE 5.9 Effect of Long-Chain n-3 Fatty Acids Fed in a Low P/S Diet on Splenocyte Nitric Oxide Production in Healthy and Tumour-Bearing Rats.** Cells were cultured without mitogen (unstimulated) or with Concanavalin A (Con A, 5 mg/L) or Phorbol Myristate Acetate (PMA, 30 µg/L) plus Ionomycin (0.75 µmol/L) for 48 h. The concentration of nitrite ($NO_2^-$) in cell culture supernatants was determined by the Griess reaction as described in Materials and Methods. Bars represent means ± SEM ($n \geq 7$/group). The effects of diet and tumour were analyzed by two-way ANOVA. For each culture condition, bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

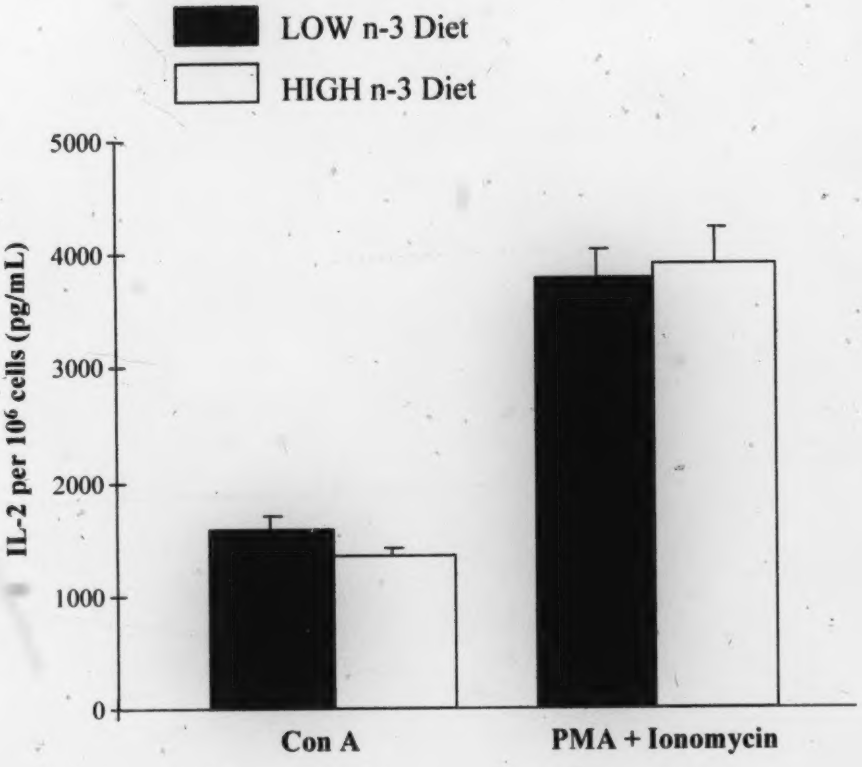

**FIGURE 5.10 Effect of Long-Chain n-3 Fatty Acids Fed in a High P/S Diet on Splenocyte Interleukin-2 Production in Tumour-Bearing Rats.** Splenocytes were cultured without mitogen (unstimulated) or with Concanavalin A (Con A, 5 mg/L) or Phorbol Myristate Acetate (PMA, 30 µg/L) plus Ionomycin (0.75 µmol/L) for 48 h. The concentration of interleukin-2 (IL-2) in splenocyte culture supernatants was determined by enzyme-linked immunosorbent assay as described in Materials and Methods. For all groups, unstimulated splenocytes produced undetectable levels of IL-2 in the culture supernatant (not shown). Bars represent means ± SEM ($n \geq 6$/diet). Diet did not significantly affect IL-2 production.

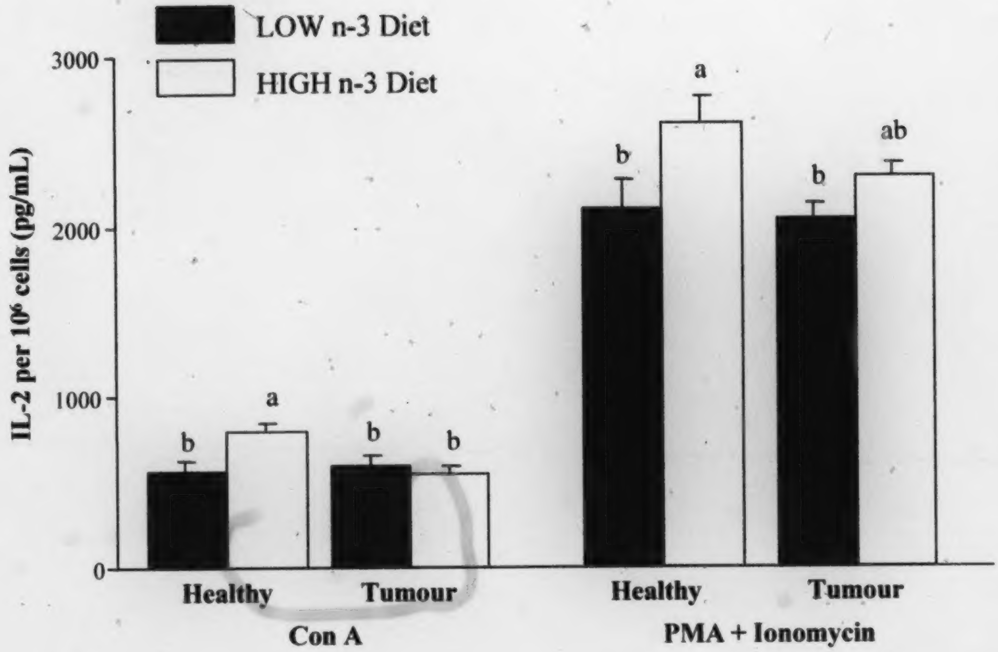

**FIGURE 5.11 Effect of Long-Chain n-3 Fatty Acids Fed in a Low P/S Diet on Splenocyte Interleukin-2 Production in Healthy and Tumour-Bearing Rats.** Splenocytes were cultured without mitogen (unstimulated) or with Concanavalin A (Con A, 5 mg/L) or Phorbol Myristate Acetate (PMA, 30 μg/L) plus Ionomycin (0.75 μmol/L) for 48 h. The concentration of interleukin-2 (IL-2) in splenocyte culture supernatants was determined by enzyme-linked immunosorbent assay as described in Materials and Methods. For all groups, unstimulated splenocytes produced undetectable levels of IL-2 in the culture supernatant. Bars represent means ± SEM ($n \geq 7$/group). The effects of diet and tumour were analyzed by two-way ANOVA. For each culture condition, bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

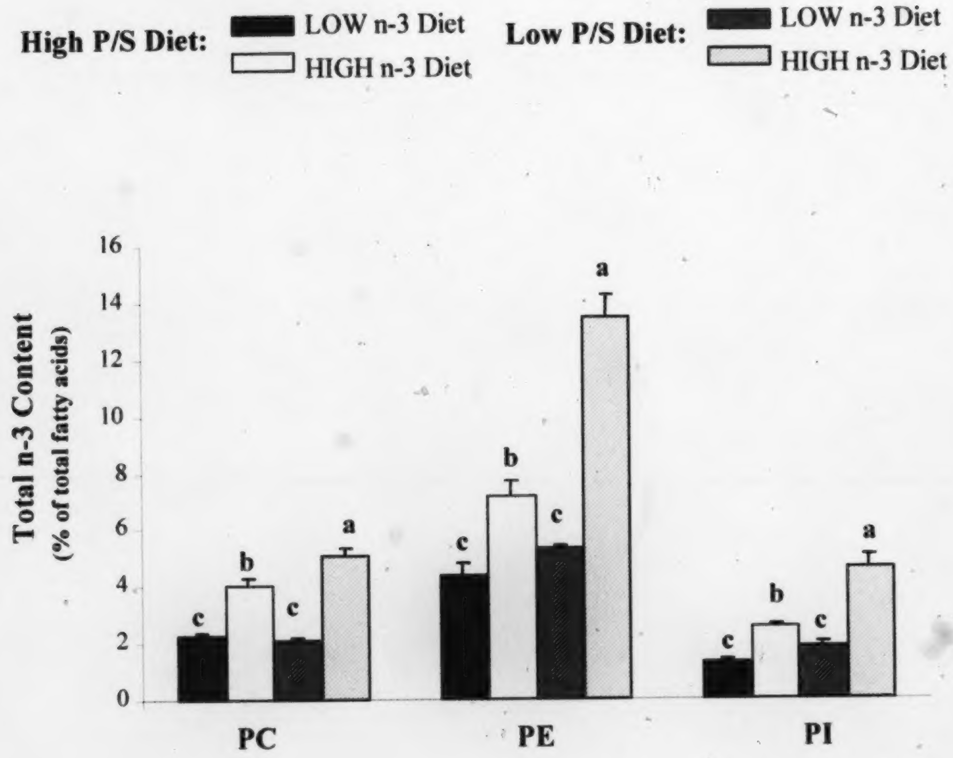

**FIGURE 5.12 Total n-3 Fatty Acid Content in Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylinositol of R3230AC Mammary Tumour Cells Isolated From Rats Fed Different Diets.** Tumour cells were isolated and lipids were extracted by a modified Folch procedure. The individual phospholipid fractions phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) were separated by thin layer chromatography and their fatty acid composition was determined by gas liquid chromatography as described in Materials and Methods. Bars represent means ± SEM ($n \geq 4$/diet). For each phospholipid, means that do not share a common letter are significantly different ($p < 0.05$) as determined by two-way ANOVA and a Duncan's multiple range test.

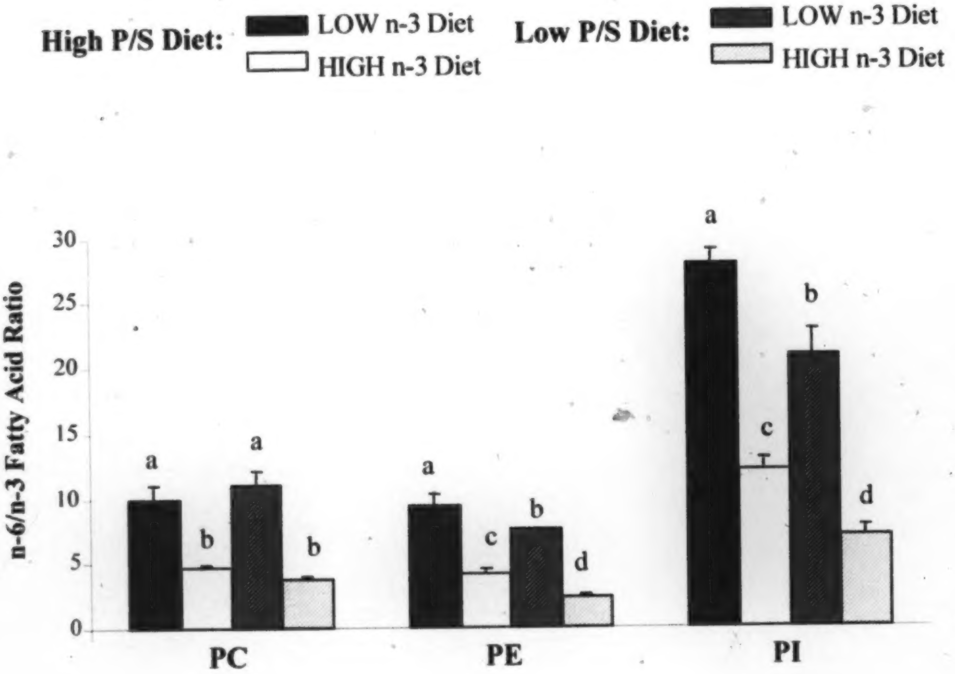

**FIGURE 5.13 n-6/n-3 Fatty Acid Ratio in Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylinositol of R3230AC Mammary Tumour Cells Isolated From Rats Fed Different Diets.** Tumour cells were isolated and lipids were extracted by a modified Folch procedure. The individual phospholipid fractions phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylinositol (PI) were separated by thin layer chromatography and their fatty acid composition was determined by gas liquid chromatography as described in Materials and Methods. Bars represent means ± SEM ($n \geq 4$/diet). For each phospholipid, means that do not share a common letter are significantly different ($p < 0.05$) as determined by two-way ANOVA and a Duncan's multiple range test.

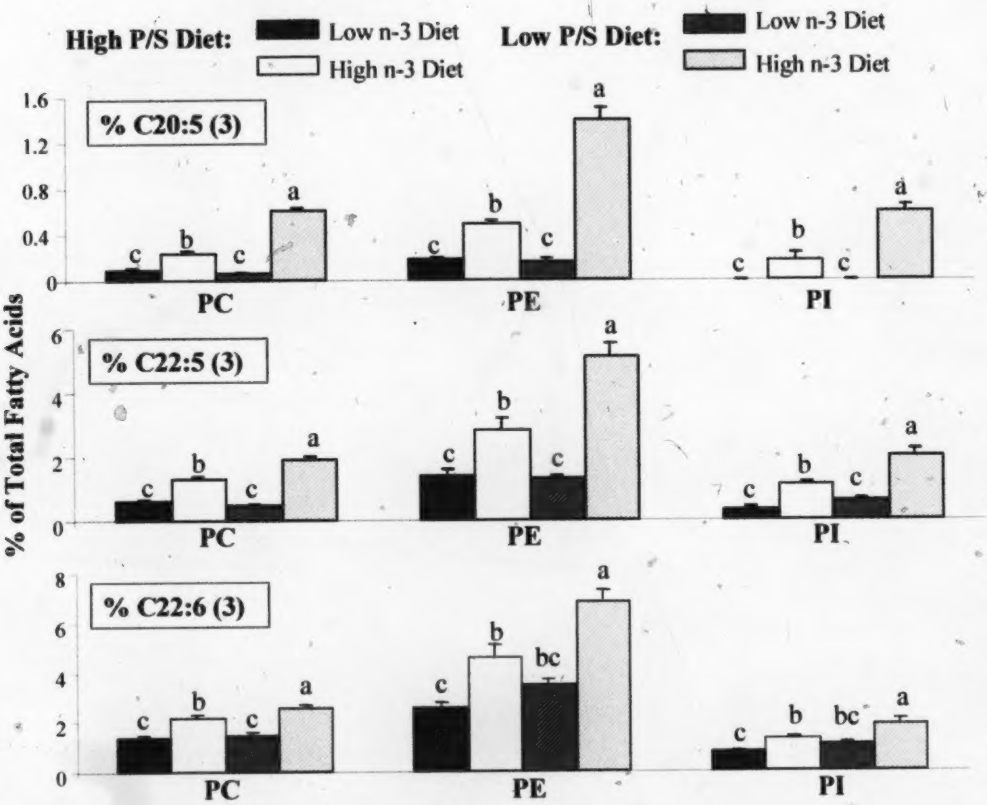

**FIGURE 5.14 Effect of Dietary n-3 Fatty Acids and P/S Ratio on C20:5(3), C22:5(3), and C22:6(3) in Phosphatidylcholine, Phosphatidylethanolamine and Phosphatidylinositol of R3230AC Mammary Tumour Cells.** Tumour cells were isolated and lipids were extracted by a modified Folch procedure. The individual phospholipid fractions phosphatidylcholine (PC), phosphatidyl-ethanolamine (PE) and phosphatidylinositol (PI) were separated by thin layer chromatography and their fatty acid composition was determined by gas liquid chromatography as described in Materials and Methods. Bars represent means ± SEM ($n \geq 4$/diet). For each phospholipid, means that do not share a common letter are significantly different ($p < 0.05$) as determined by two-way ANOVA and a Duncan's multiple range test.

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# 6. INFLUENCE OF THE R3230AC MAMMARY TUMOUR AND DIET ON HOST IMMUNITY: CHANGES IN IMMUNE CELL COMPOSITION AND FUNCTION DURING MITOGEN ACTIVATION[1]

## 6.1 INTRODUCTION

Anticancer immune defense involves components of both the innate (natural) and acquired (specific) cell-mediated immune systems, including natural killer (NK) cells, macrophages, $CD4^+$ T helper (Th) and $CD8^+$ T suppressor/cytotoxic cells (Whiteside and Herberman 1995, Robins 1986, Adams et al. 1982). Since anticancer immunity declines progressively with tumour growth (Kiessling et al. 1999, Shewchuk et al. 1996), a major focus of current research in tumour immunology is the development of means to stimulate the host immune system. For example, specific dietary nutrients have received considerable attention for their potential immunoenhancing properties (Atkinson et al. 1998, Weimann et al. 1998).

Diets rich in fish oil-derived long-chain n-3 fatty acids, eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids, can affect components of both the innate and acquired cell-mediated immune systems (Calder 1998). The mechanism for the immunomodulatory effect of n-3 fatty acids may involve changes in membrane-mediated functions through alterations in membrane lipid composition (Calder 1998, Peck 1994). It is well established that changes in dietary fat composition can induce significant alterations in the composition and function of membranes in a variety of human and animal tissues (Field et al. 1988, Clandinin et al. 1985), including immune cells (Field et al. 2000, Peterson et al. 1998, Hosack-Fowler et al. 1993, Tiwari et al. 1987). In lymphocytes, membrane-associated events play a pivotal role in signal transduction (Hosack-Fowler et al 1993), the expression of surface markers (Jenski et al. 1995, Yaqoob et al. 1994) and cellular activation (Calder et al. 1994, Hadden 1988), all of which are important in immune cell function. Also, many functions of the immune system depend on interactions between effector and target cell membranes. Thus, changing the membrane composition of such cells, through modulating dietary lipids, may influence immune responses important in anticancer defense. Less is known about how the lipid composition of immune cell membranes is altered during tumour growth, when immune cells are activated or suppressed by the presence of the tumour. It is possible that tumour-induced suppression of various host immune responses may be associated with changes in the lipid composition of immune cell membranes. Furthermore, it is not currently known how dietary fat and tumour growth interact to affect immune cell membrane composition and function. The objectives of this study were to determine the effects of dietary long-chain n-3 fatty acids and tumour burden on immune cell membrane phospholipid composition and membrane-mediated immune defense in rats implanted with the R3230AC mammary adenocarcinoma. Specifically, we hypothesized that suppression of membrane-mediated immune defense during tumour growth is associated with changes in immune cell membrane composition.

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[1] A version of this chapter has been submitted for publication to *Journal of Nutrition*.

## 6.2 MATERIALS AND METHODS

**Animals and diets.** Experiments were reviewed and approved by the Faculty of Agriculture and Forestry Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Thirty-three female Fischer 344 rats (145 ± 2 g) were obtained from a colony maintained at the University of Alberta and were housed in individual wire-mesh cages in a temperature controlled room (23 °C) maintained on a 12 h light/dark cycle. Body weight and food intake were recorded every third day throughout the study. Rats were randomly assigned to be fed nutritionally complete, semi-purified diets (Teklad Test Diets, Madison, WI) containing (per kg) 270 g high protein casein, 408 g carbohydrate and 200 g fat. Both diets met the n-6 and n-3 fatty acid requirements of a growing rat. The complete nutrient composition of the diets was described in Chapter 3. The dietary P/S ratio was 0.35 as determined by gas-liquid chromatography (Field et al 1988). The two diets differed only in the composition of fat, providing two different levels of long chain n-3 fatty acids from a mixed fish oil source (P-28 Nisshin lot # 28020, Nisshin Flour Milling Co., Ltd., Tokyo, Japan): low (0 g/kg) or high (50 g/kg of total fat). The only source of n-3 fatty acids in the low n-3 diet was α-linolenic acid (C18:3n-3), provided by linseed oil (Galaxy Enterprises, Edmonton, Canada). The fatty acid composition of the diets is presented in **Table 6.1**. All animals were given free access to food and water. After 21 d of feeding, a freshly harvested R3230AC mammary tumour from a rat implanted 2-3 wk earlier was finely chopped under sterile conditions to prepare a tumour brei and 50 μL was injected subcutaneously in the inguinal region of experimental rats. Rats were killed by $CO_2$ asphyxiation and cervical dislocation 17 d following tumour implantation. At necropsy, tumour and spleen were removed and weighed to be used for the measurements described below. There were 14 tumour-bearing rats (7/diet) and 19 healthy (control) rats (9 rats were fed the low n-3 diet and 10 rats were fed the high n-3 diet).

**Splenocyte isolation and activation.** Splenocytes were isolated aseptically in Krebs-Ringer HEPES buffer (KRH, pH 7.4) supplemented with bovine serum albumin (5 g/L, Sigmal Chemical, St. Louis, MO) as described in Chapter 3. Isolated splenocytes (3.0 x $10^9$ cells/L) in complete culture media (CCM; defined in Chapter 3) were incubated in 24-well sterile plates for 48 h in a humidified atmosphere at 37 °C in the presence of 5% $CO_2$. The cell culture media contained either no mitogen (unstimulated cells) or was supplemented with either Con A (5 mg/L) or PMA (30 μg/L) plus Ionomycin (0.75 μmol/L). After 48 h, splenocyte culture supernatants were collected and stored at −70 °C for subsequent cytokine analysis. Unstimulated and stimulated splenocytes were washed twice in KRH (pH 7.4) supplemented with bovine serum albumin (5 g/L) and used for either indirect immunofluorescence analyses or frozen at −70 °C for subsequent lipid analysis.

**Splenocyte mitogenic response (proliferation) assay.** Splenocytes (1.25 x $10^9$ cells/L) were cultured in triplicate in 96-well microtiter plates (Corning Glass Works, Corning, NY) in complete culture media with or without either Con A (5 mg/L) or PMA (30 μg/L) plus Ionomycin (0.75 μmol/L) for 66 or 78 h as described in Chapter 4. Twelve hours prior to harvesting the cells, each well was pulsed with 18.5 kBq of [methyl-$^3$H]-thymidine (Amersham, Oakville, ON, Canada).

**Indirect immunofluorescence (phenotype) assay**. Immune cell subsets in cultured and mitogen-stimulated splenocytes were identified by indirect immunofluorescence assay as described in Chapter 3. The monoclonal antibodies used were the same as those defined in Chapters 3 and 5 (**Table 6.2**).

**Cytokine production**

**Interferon-gamma (IFN-γ).** The concentration of IFN-γ in culture supernatants collected from unstimulated and stimulated splenocytes was determined by a colorimetric ELISA (all antibodies and standards were purchased from Genzyme Diagnostics, Cambridge, MA). Supernatants were prepared and frozen at –70 °C as described above. Purified anti-cytokine capture antibody was diluted to 2 μg/mL in coating buffer (see Appendix B for composition of all solutions in this protocol) and 100 μL was added to each well of flat-bottom microtiter plates (Immulon®, Dnyex Technologies Inc., Chantilly, VA). Plates were sealed, incubated overnight at 4 °C, and washed 3 times with wash buffer. Each wash involved adding 200 μL of wash buffer, gently shaking for 1 min, and inverting and blotting the plate on an absorbent surface. Non-specific binding was blocked by adding 250 μL of blocking buffer/well, sealing the plates and incubating at 37 °C for 2 h. Plates were then washed 3 times as described above. Recombinant IFN-γ standard was reconstituted in $ddH_2O$ to a concentration of 4.86 ng/mL. Standards were diluted in blocking buffer and 100 μL was added to each well (20-1620 pg/mL). Stimulated samples were added (25 μL/well) and diluted with 75 μL of blocking buffer, whereas unstimulated samples were added (50 μL/well) and diluted with 50 μL of blocking buffer so that all wells contained equal volumes and to ensure that sample concentrations were within the linear portion of the standard curve. Differences in the amount of supernatant added were accounted for in the final calculations. Standards and samples were added to wells in duplicate. Plates were then sealed, incubated for 2 h at 37 °C and washed 4 times. Biotinylated anti-IFN-γ detecting antibody was diluted to 1.25 μg/mL in blocking buffer and 100 μL was added to each well. Plates were sealed, incubated at 37 °C for 1 h and washed 4 times. Horseradish peroxidase-streptavidin was diluted 1:10000 in blocking buffer and 100 μL/well was added, plates were sealed, incubated at 37 °C for 30 min, and washed 4 times. Tetramethylbenzidine substrate solution (Sigma Chemical) was thawed immediately prior to use and 100 μL was added to each well. Plates were incubated at room temperature for approximately 10 min at which time 50 μL of stop solution was added. The optical density was read at 450 nm on a microplate reader (Model EL309; Bio-tek Instruments Inc., Burlington, VT). The concentration of IFN-γ in splenocyte culture supernatants (unstimulated and stimulated) was quantified by comparison to the standard curve generated with recombinant IFN-γ.

**Tumour Necrosis Factor-alpha (TNF-α).** The concentration of TNF-α in culture supernatants collected from unstimulated and stimulated splenocytes was determined by a colorimetric ELISA (all antibodies and standards were purchased from R&D Systems, Minneapolis, MN). Supernatants were prepared and frozen at –70 °C as described above. Purified anti-cytokine capture antibody was diluted to 2 μg/mL in PBS (see Appendix B for composition of all solutions in this protocol) and 100 μL was added to each well of flat-bottom microtiter plates (Immulon®). Plates were sealed, incubated overnight at room temperature, and washed 3 times with wash buffer. Each wash involved adding 200 μL of wash buffer, gently shaking for 1 min, and inverting and blotting the plate on an absorbent surface. Non-specific binding was blocked by adding 250 μL of blocking buffer/well, sealing the plates and incubating at room temperature for 1 h. Plates were then washed 3 times as described above. Recombinant TNF-α standard was reconstituted in diluent to various concentrations (15-2000 pg/mL). Standards and samples were then added (100 μL/well) in duplicate. Plates were sealed, incubated for 2 h at room temperature and washed 3 times with wash buffer. Biotinylated anti-cytokine detecting antibody was diluted to 200 ng/mL in diluent and 100 μL was added to each well. Plates were sealed, incubated at room temperature for 2 h and washed 3 times with wash buffer. Horseradish peroxidase-streptavidin was diluted in blocking buffer (0.5 μL/mL) and 100 μL was added to each well, plates were sealed, incubated at room temperature for 30 min, and washed 8 times. ABTS Substrate Solution was thawed 20 min prior to use and 10 μL of 30% $H_2O_2$/11 mL of substrate was added and vortexed. Then, 100 μL of this solution was added to each well and plates were incubated at room temperature for 30 min. The optical density was read at 405 nm on a microplate reader (Model EL-309; Bio-Tek Instruments Inc.) and the concentration of TNF-α in splenocyte culture supernatants (unstimulated and stimulated) was quantified by comparison to the standard curve generated with recombinant TNF-α.

**Splenocyte fatty acid analysis.** Lipids were extracted from splenocytes by a modified Folch (Folch et al. 1957) procedure (Field et al 1988) as described in Chapter 3. Individual phospholipids were separated on thin layer chromatography plates (HPK silica gel 60 Å 10 x 10 cm, Whatman, Clifton, NJ) as previously described (Touchstone et al. 1980). Separated phospholipids were visualized with 8-anilino-1-naphthalene-sulfonic acid and identified under ultraviolet light with appropriate standards. Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) fatty acid methyl esters were prepared from the scraped silica band using 14 % (w/v) $BF_3$/methanol reagent (Morrison and Smith 1964) and separated by automated gas liquid chromatography (Vista 6010, Varian Instruments, Georgetown, ON) on a fused silica BP20 capillary column (25 m x 0.25 mm internal diameter, Varian Instruments) as previously described (Field et al 1988).

**Statistical analysis.** Results are presented as means ± SEM. All statistical analyses were conducted using the SAS statistical package (Version 6.11, SAS Institute, Cary, NC). The effects of diet and tumour were determined by two-way ANOVA followed by a Duncan's multiple range test to identify significant ($p \leq 0.05$) differences between individual treatments (Steele and Torrie 1980). Body weight changes and food intake were compared among groups by a split-plot (repeated measures) ANOVA (Steele and Torrie 1980). Paired *t* tests were used to compare cytokine production by immune cells with or without mitogen.

## 6.3 RESULTS

**Food intake, body weight and spleen weight.** Neither dietary long-chain n-3 fatty acids nor the tumour significantly affected food intake (63 ± 1g/kg body weight/d, *n* = 29), final body weight (158 ± 2 g, $n = 33$), weight increase (13 ± 1 g, $n = 33$), relative spleen weight (2.8 ± 0.1 g/kg body weight, $n = 33$), or the number of spleen cells (x $10^6$) isolated per gram of spleen (433 ± 13, $n = 32$).

**R3230AC mammary tumour weight.** Final tumour weight was lower (-31 %) when rats were fed long-chain n-3 fatty acids (1.3 ± 0.2 vs. 0.9 ± 0.1 g/100 g body weight, $n =$ 7/diet), but this did not reach statistical significance ($p = 0.1$).

**Splenocyte mitogenic response.** Neither diet nor the tumour affected [$^3$H]-thymidine incorporation by unstimulated splenocytes at 66 and 78 h (66 h, 2422 ± 251 Bq/min, $n =$ 33; 78 h, 1898 ± 244 Bq/min, $n = 33$) (**Table 6.3**). Diet did not significantly affect [$^3$H]-thymidine uptake by either Con A or PMA plus Ionocmycin stimulated cells at 66 and 78 h; therefore, rats in the low and high n-3 diet groups within either the healthy or tumour-bearing group were combined for statistical analysis to examine the effect of tumour burden (**Figure 6.1**). Splenocytes from tumour-bearing rats had a lower response to both Con A and PMA plus Ionomycin at 66 and 78 h ($p < 0.01$) compared with cells from healthy rats (Figure 6.1).

**Immune Cell Phenotypes in Spleen Post-Culture**

**(a) Unstimulated.** Diet did not significantly affect immune cell phenotypes in splenocytes from healthy and tumour-bearing rats after cells were cultured for 48 h without mitogen (unstimulated) (**Table 6.4**); therefore, rats in the low and high n-3 diet groups within either the healthy or tumour-bearing group were combined for statistical analysis to examine the effect of tumour burden (**Table 6.5**). Splenocytes from tumour-bearing rats had a significantly higher relative proportion (% of total cells) of CD5$^+$ total T cells, CD4$^+$ Th cells, CD8$^+$ T suppressor/cytotoxic cells, CD28$^+$ cells, CD25$^+$ cells, and CD4/CD8 ratio compared with healthy rats (Table 6.5). In addition, tumour-bearing rats had a significantly lower proportion of B cells compared with healthy rats (Table 6.5). The proportion of macrophages was higher (+48%) in tumour-bearing rats, but this did not reach significance ($p < 0.09$, Table 6.5).

**(b) After 48 h PMA plus Ionomycin stimulation.** There was a significant ($p \leq 0.01$) diet by tumour interaction on the proportion of $CD8^+$ T suppressor/cytotoxic cells after splenocytes were stimulated with PMA plus Ionomycin (**Table 6.6**). In the high n-3 diet group, tumour-bearing rats had a significantly higher proportion of $CD8^+$ T suppressor/cytotoxic cells and a significantly lower CD4/CD8 ratio compared with healthy rats fed the same diet (Table 6.6). Tumour burden did not significantly affect the proportion of $CD8^+$ T suppressor/cytotoxic cells or the CD4/CD8 ratio when rats were fed the low n-3 diet (Table 6.6). In both diet groups, splenocytes from tumour-bearing rats had a significantly lower proportion of $CD4^+$ Th cells and B cells after mitogen stimulation compared with healthy rats (Table 6.6). Neither diet nor the tumour significantly affected the proportion of macrophages or $CD28^+$ cells after mitogen stimulation (Table 6.6).

**IL-2 receptor (CD25) expression after 48 h PMA plus Ionomycin stimulation.** There was a significant ($p \leq 0.01$) diet by tumour interaction on the proportion of $CD25^+$ $CD8^+$ T suppressor/cytotoxic cells after splenocytes were stimulated with PMA plus Ionomycin (**Figure 6.2**). In the high n-3 diet group, tumour-bearing rats had a significantly higher proportion of $CD25^+$ $CD8^+$ T suppressor/cytotoxic cells compared with healthy rats fed the same diet (Figure 6.2). Tumour burden did not significantly affect the proportion of $CD25^+$ $CD8^+$ T suppressor/cytotoxic cells when rats were fed the low n-3 diet (Figure 6.2). Tumour-bearing rats fed the high n-3 diet had a significantly higher proportion of $CD25^+$ $CD8^+$ T suppressor/cytotoxic cells compared with those fed the low n-3 diet (Figure 6.2). Diet did not significantly affect $CD25^+$ expression on other cell types (**Table 6.7**); therefore, groups were combined (**Figure 6.3**). Tumour-bearing rats had a significantly lower proportion of $CD25^+$ $CD4^+$ Th cells, $CD25^+$ B cells, $CD25^+$ macrophages and $CD25^+$ $CD28^+$ cells after mitogen stimulation compared with healthy rats (Figure 6.3).

### Cytokine Production

**(a) IFN-γ.** For all groups, splenocytes cultured for 48 h without mitogen (unstimulated) produced undetectable levels of IFN-γ in the culture supernatants (results not shown).

Dietary long-chain n-3 fatty acids did not affect IFN-γ production by splenocytes stimulated with Con A (**Table 6.8**). In both diet groups, tumour-bearing rats produced significantly more IFN-γ after Con A stimulation, compared with healthy rats (Healthy rats, 841 ± 52 pg/mL/$10^6$ cells, *n* = 13; Tumour-bearing rats, 993 ± 46 pg/mL/$10^6$ cells, *n* = 12) (Table 6.8). In tumour-bearing rats, IFN-γ production by splenocytes stimulated with PMA plus Ionomycin was significantly higher when rats were fed the high n-3 diet compared with the low n-3 diet (**Figure 6.4**). The tumour did not significantly affect IFN-γ production by splenocytes stimulated with PMA plus Ionomycin (Figure 6.4).

**(b) TNF-α.** Neither diet nor the tumour significantly affected TNF-α production by splenocytes cultured for 48 h without mitogen (31 ± 6 pg/mL/$10^6$ cells, *n* = 24). (Table 6.8). Dietary long-chain n-3 fatty acids did not affect TNF-α production by splenocytes stimulated with Con A (Table 6.8). In both diet groups, tumour-bearing rats produced significantly more TNF-α after Con A stimulation compared with healthy rats (Tumour-bearing, 106 ± 6 pg/mL/$10^6$ cells, *n* = 10; Healthy, 75 ± 9 pg/mL/$10^6$ cells, *n* = 12) (Table 6.8). There was a significant diet by tumour interaction ($p \leq 0.01$) on splenocyte TNF-α production after PMA plus Ionomycin stimulation (Figure 6.4). In both healthy and tumour-bearing rats, TNF-α production by PMA plus Ionomycin-stimulated splenocytes was increased ($p < 0.0001$) when rats were fed the high n-3 diet compared with the low n-3 diet (Figure 6.4). Furthermore, tumour-bearing rats fed the high n-3 diet produced more ($p < 0.0001$) TNF-α compared with healthy rats fed the same diet (Figure 6.4).

### Fatty Acid Composition of Immune Cells

The proportions of fatty acids from C14:0 to C24:1(9) in PC and PE were measured, but only major fatty acids are reported (**Tables 6.9-6.14**).

### (1) Freshly Isolated Splenocytes

**PC.** Freshly isolated splenocytes from both healthy and tumour-bearing rats fed the high n-3 diet had a significantly higher % of C18:2(6), C20:5(3), and C22:5(3) and total n-3 polyunsaturated fatty acid content in PC in freshly isolated splenocytes compared with those fed the low n-3 diet (**Table 6.9**). In addition, both healthy and tumour–bearing rats fed the high n-3 diet had a significantly lower % of C20:4(6), C22:4(6) and n-6/n-3 fatty acid ratio in PC compared with those fed the low n-3 diet (Table 6.9). For healthy rats, those fed the high n-3 diet had a significantly lower % of C20:2(6) in splenocyte PC compared with those fed the low n-3 diet (Table 6.9). However, there was no effect of diet on the % of C20:2(6) in splenocyte PC from tumour-bearing rats (Table 6.9). Diet significantly affected the % of C22:5(6) and C22:6(3) in PC in freshly isolated splenocytes from tumour-bearing rats, but not healthy rats (Table 6.9). Specifically, tumour-bearing rats fed the high n-3 diet had a significantly lower and higher % of C22:5(6) and C22:6(3), respectively, compared with those fed the low n-3 diet (Table 6.9). The tumour significantly affected total n-3 fatty acids and n-6/n-3 fatty acid ratio in PC, but only in rats fed the low n-3 diet. Specifically, in the low n-3 diet group, tumour-bearing rats had a significantly lower % of C20:5(3) and total n-3 fatty acid content and a significantly higher n-6/n-3 fatty acid ratio in PC compared with healthy rats (Table 6.9). In contrast, the tumour did not significantly affect n-3 fatty acids or the n-6/n-3 fatty acid ratio in splenocyte PC when rats were fed the high n-3 diet (Table 6.9). The total saturated and monounsaturated fatty acid content and P/S ratio in splenocyte PC were not significantly affected by either dietary long-chain n-3 fatty acids or the tumour (Table 6.9).

**PE.** Freshly isolated splenocytes from both healthy and tumour-bearing rats fed the high n-3 diet had a significantly higher % of C18:2(6), C20:5(3), C22:5(3), C22:6(3) and total n-3 fatty acid content in PE compared with those fed the low n-3 diet (**Table 6.10**). In addition, both healthy and tumour-bearing rats fed the high n-3 diet had a significantly lower % of C22:4(6) and C22:5(6), total n-6 fatty acids and n-6/n-3 ratio in splenocyte PE compared with those fed the low n-3 diet (Table 6.10). The % of C20:4(6) in PE was also significantly lower in rats fed the high n-3 diet, but this diet effect was only present in healthy, not tumour-bearing, rats (Table 6.10). The only fatty acid in PE that was significantly affected by the tumour was C22:5(6). The % of C22:5(6) in splenocyte PE was significantly increased in tumour-bearing rats compared with healthy rats, but only in rats fed the low n-3 diet (Table 6.10). The total saturated and monounsaturated fatty acid content and P/S ratio in splenocyte PE were not significantly affected by either dietary long-chain n-3 fatty acids or the tumour (Table 6.10).

### (2) Splenocytes Post-Culture

### (a) After 48 h Con A Stimulation

**PC.** Both healthy and tumour-bearing rats fed the high n-3 diet had a significantly higher % of C18:2(6) in PC in splenocytes stimulated with Con A (**Table 6.11**). The % of C18:1(9) (and total monounsaturated fatty acids) and C22:6(3) (and total n-3 fatty acids) in PC was significantly increased when healthy rats were fed the high n-3 diet compared with the low n-3 diet, but this diet effect was not present in tumour-bearing rats (Table 6.11). The % of C20:2(6) and C22:4(6) was significantly decreased when tumour-bearing rats were fed the high n-3 diet compared with the low n-3 diet, but this effect was not present in healthy rats (Table 6.11). Overall, the tumour had minimal effects on the fatty acid composition of PC in splenocytes stimulated with Con A. Specifically, tumour-bearing rats had a significantly higher % of C18:1(9) in PC compared with healthy rats, but only in the low n-3 diet group (Table 6.11). In contrast, the tumour did not significantly affect the % of C18:1(9) when rats were fed the high n-3 diet (Table 6.11). Tumour-bearing rats had a significantly lower % of C20:2(6) and a significantly higher % of C20:3(9) in PC compared with healthy rats, but only in the high n-3 diet group (Table 6.11). Neither diet nor the tumour significantly affected total saturated or n-6 fatty acids or the n-6/n-3 or P/S ratios in PC in splenocytes stimulated with Con A (Table 6.11).

**PE.** Neither dietary long-chain n-3 fatty acids nor the tumour significantly affected the fatty acid composition of PE in splenocytes stimulated with Con A with the following exception: tumour-bearing rats fed the high n-3 diet had a significantly increased % of C20:2(6) compared with healthy rats fed the same diet or those fed the low n-3 diet (**Table 6.12**).

### (b) After 48 h PMA plus Ionomycin Stimulation

**PC.** Both healthy and tumour-bearing rats fed the high n-3 diet had an increased % of C22:5(3) and total n-3 fatty acids in PC in splenocytes stimulated with PMA plus Ionomycin compared with those fed the low n-3 diet (**Table 6.13**). Healthy rats fed the high n-3 diet also had a significantly higher % of C18:1(7) and a significantly lower % of C20:2(6), C22:5(6) and C22:4(6) and n-6/n-3 fatty acid ratio in PC compared with those fed the low n-3 diet (Table 6.13). However, diet did not affect the proportion of these fatty acids in tumour-bearing rats. The tumour only affected the fatty acid composition of PC in splenocytes stimulated with PMA plus Ionomycin when rats were fed the low n-3 diet. For example, low n-3-fed tumour-bearing rats had a significantly higher % of C18:1(7) and a significantly lower % of C20:2(6), C20:3(6) and C22:4(6) and total n-6 fatty acids and n-6/n-3 fatty acid ratio in PC compared with healthy rats fed the same diet (Table 6.13). Neither diet nor the tumor significantly affected the total saturated or monounsatured fatty acid content or P/S ratio in PC in splenocytes stimulated with PMA plus Ionomycin (Table 6.13).

**PE.** Both healthy and tumour-bearing rats fed the high n-3 diet had a significantly lower % of C20:1(9) and n-6/n-3 fatty acid ratio in PE in splenocytes stimulated with PMA plus Ionomycin (**Table 6.14**). Healthy rats fed the high n-3 diet also had a significantly lower % of C20:2(6) compared with those fed the low n-3 diet (Table 6.14). Tumour-bearing rats fed the high n-3 diet had a significantly lower % of C16;1(7) and a significantly higher % of C20:0, C20:5(3), C22:5(3), C22:6(3) and total n-3 fatty acids compared with those fed the low n-3 diet (Table 6.14). The tumour only affected the fatty acid composition of PE in splenocytes stimulated with PMA plus Ionomycin when rats were fed the high n-3 diet. For example, high n-3-fed tumour-bearing rats had a significantly higher % of C18:0, C22:5(3) and C22:6(3) and total n-3 fatty acids, and a significantly lower monounsaturated fatty acid content in PE compared with healthy rats fed the same diet (Table 6.14). Neither diet nor the tumor significantly affected the total saturated or n-6 fatty acids or P/S ratio in PE in splenocytes stimulated with PMA plus Ionomycin (Table 6.14).

## 6.4 DISCUSSION

The lipid composition of a cell membrane is a major determinant of its physical properties and also modulates important membrane-mediated cell functions such as integral enzyme activity, membrane receptor function and eicosanoid production (Clandinin et al 1985). Since many functions of the immune system depend on interactions between effector and target cell membranes, changing the membrane composition of such cells may influence immune responses. *In vitro* stimulation with mitogens such as Con A or a combination of PMA plus Ionomycin, provides a useful tool for studying immune cells in an activated state. Con A is a lectin which binds specifically to certain sugar residues on T cell surface glycoproteins, including the T cell receptor and CD3, thereby stimulating T cells (Fleischer 1984). PMA, which activates protein kinase C, and Ionomycin, a $Ca^{2+}$ ionophore which increases intracellular calcium concentration, act together to stimulate proliferation of a variety of cell types, including T and B cells (Berry et al. 1989, Truneh et al. 1985). In this study, we determined the fatty acid composition of membrane phospholipids in freshly isolated (resting) and mitogen-activated splenocytes from healthy and tumour-bearing rats. We also investigated if alterations in immune cell membrane composition were related to changes in host immune function and the effect of feeding fish oil-derived long-chain n-3 fatty acids on these properties.

This study confirms previous findings that dietary fat significantly alters the fatty acid composition of immune cell phospholipids (Field et al 2000, Peterson et al 1998, Hosack-Fowler et al 1993). As expected, freshly isolated splenocytes from both healthy and tumour-bearing rats fed the high n-3 diet had a significantly higher n-3 fatty acid content and a significantly lower n-6/n-3 ratio in PC and PE (Tables 6.9-6.10). We have previously shown that many diet-induced changes in immune cell phospholipid composition are maintained 48 h post-culture when splenocytes are incubated either in the absence or presence of Con A (Chapter 4). In the present study, maintenance of diet-induced alterations in splenocyte phospholipid composition during activation varied with the presence of a tumour, the mitogen used to stimulate splenocytes, and the individual phospholipid fraction. For example, the increase in n-3 fatty acids in PC in rats fed the high n-3 diet was maintained in the Con A-activated state, but only in healthy, not tumour-bearing, rats (Table 6.11). Similarly, the lower n-6/n-3 ratio in PC in high n-3-fed rats was maintained after PMA plus Ionomycin activation, but only in healthy rats (Table 6.13). Thus, diet-induced changes in n-6 and n-3 fatty acids were better maintained after activation in healthy rats. As well, the high n-3 diet-induced increase in n-3 fatty acids in PE was maintained in PMA plus Ionomycin-stimulated splenocytes from tumour-bearing rats (Table 6.14). Overall, freshly isolated splenocyte membrane lipids were more reflective of dietary lipids than membranes post mitogen-activation, especially in tumour-bearing rats. This may have been partially due to the effect of mitogen activation on membrane composition (Calder et al 1994, Goppelt-Strübe and Resch 1987), and potential differences between healthy and tumour-bearing rats in cell activation (Fujiwara and Hamaoka 1995), but this could not be assessed in the current study since we lacked lipid data from post-culture (unstimulated) cells to use as a proper comparison. Furthermore, in our previous work, long-chain n-3 fatty acids fed in a high polyunsaturated/saturated fat (P/S) diet suppressed cell proliferation in healthy rats (Chapter 4), whereas there was no effect of long-chain n-3 fatty acids fed in a low P/S diet on cell proliferation in the present study (Table 6.3). Overall, the influence of long-chain n-3 fatty acids on membrane composition of activated splenocytes may have varied due to tumour burden, dietary composition, and activation state of the immune cells.

It is known that various human cancers result in changes in the composition of host tissues (Baro et al. 1998, Engan et al. 1995, Mosconi et al. 1989). For example, colorectal cancer patients have abnormal plasma and erythrocyte fatty acid profiles characterized by decreased levels of most saturated, monounsaturated, and essential fatty acids, as well as their polyunsaturated metabolites (Baro et al 1998). It has been suggested that such abnormalities are likely due to metabolic changes induced by the cancer *per se*, as opposed to malnutrition (Baro et al 1998). Less is known about alterations in membrane lipid composition of other host tissues, such as immune cells, during mammary tumour growth when host malnutrition is not necessarily present. Furthermore, it is not currently known whether dietary fat composition and tumour growth interact to affect immune cell membrane composition. Interestingly, we found that some of the tumour-induced effects on immune cell phospholipids differed with dietary fat composition. For example, the R3230AC mammary tumour significantly altered n-3 and n-6 fatty acid levels in PC in freshly isolated splenocytes and PMA plus Ionomycin-activated cells, but only in the low n-3 diet group (Table 6.9 and 6.13). In contrast, the tumour increased the n-3 fatty acid content in PE in splenocytes stimulated with PMA plus Ionomycin, but only when rats were fed the high n-3 diet (Table 6.14).

Tumour immunity is mediated to a large extent by activated CD8$^+$ cytotoxic T lymphocytes (CTLs) that induce apoptotic death in tumour cells (Kiessling et al 1999) and whose activation and function is supported by type-1 cytokines, such as IL-2, IFN-$\gamma$ and TNF-$\alpha$ (Pardoll and Topalian 1998), produced by CD4$^+$ Th1 cells and CD8$^+$ T cells (Mosmann and Sad 1996). It is possible that tumour-induced changes in membrane composition of immune cells subsequently affects immune responses in tumour-bearing hosts. In the present study, tumour-bearing rats had a higher proportion of CD5$^+$ T cells (CD4$^+$ Th cells and CD8$^+$ cells) and CD4/CD8 ratio, as well as an increased expression of the IL-2 receptor (CD25) activation marker and the T cell costimulatory molecule, CD28, on the surface of splenocytes cultured without mitogen (Table 6.5). T cell activation requires at least two independent signals, one via the T cell receptor and a second via CD28 which provides a signal critical to IL-2 production and T cell proliferation (June et al. 1994). Although our findings suggest that splenocytes (cultured without mitogen) from tumour-bearing rats are more primed for T cell-mediated responses, the tumour did not significantly affect unstimulated splenocyte proliferation (Table 6.3) or cytokine production (Table 6.8), suggesting a functional impairment in these animals. Furthermore, after splenocytes were stimulated *in vitro* (with PMA plus Ionomycin), tumour-bearing rats had a lower proportion of activated (CD25$^+$) CD4$^+$ Th cells, macrophages, CD28$^+$ cells, and B cells (Figure 6.3) which was accompanied by suppressed [$^3$H]-thymidine incorporation by splenocytes (Figure 6.1). Thus, our results show that tumour-bearing rats did not respond as well to mitogen stimulation compared with healthy rats, supporting that there is impaired cell-mediated immune function in these rats at 17 d post-implantation. While there is limited data on the effect of the R3230AC mammary tumour on host immune function, other work supports suppressed host immunity with tumour growth (Kiessling et al 1999, Shewchuk et al 1996). Although energy restriction alters both tumour growth (Kritchevsky 1990) and immune function (Corman 1985), it is unlikely that the observed immune suppression was due to energy malnutrition since tumour-bearing rats had similar weight gain and food intake compared with control (healthy) rats at 17 d post-implantation.

Despite the observed decrease in activation marker expression and splenocyte proliferation in tumour-bearing rats, production of type-1 cytokines, IFN-γ and TNF-α, was higher in tumour-bearing rats, although there were some differences with the mitogen used (Table 6.8 and Figure 6.4). Although the proportion of activated $CD4^+$ Th cells, which produce IFN-γ and TNF-α, was decreased with tumour growth, we cannot determine if there was a potential shift in $CD4^+$ Th1 and Th2 cell subsets during tumour growth which may have altered cytokine production. It is possible that more $CD4^+$ Th1 versus Th2 cells were present in spleen at 17 d post-implantation, resulting in increased IFN-γ and TNF-α in tumour-bearing rats. As well, these type-1 cytokines are produced by other cell types such as $CD8^+$ T cells, NK cells and macrophages (Mosmann and Sad 1996). In the case of tumour-bearing rats fed the high n-3 diet, the increased proportion of activated $CD8^+$ T cells may have accounted for the higher levels of IFN-γ and TNF-α produced.

A major focus of current research in immunology and oncology is the development of methods to augment host antitumour immune defense. Our goal was to determine if dietary fish-oil derived long-chain n-3 fatty acids could enhance membrane-mediated immune function in rats implanted with the R3230AC mammary adenocarcinoma. Although dietary long-chain n-3 fatty acids were not able to reverse the suppressed proliferative response to mitogen in tumour-bearing rats (Table 6.3), there was a significantly higher proportion of activated ($CD25^+$) $CD8^+$ T cells (Figure 6.2) and increased IFN-γ and TNF-α production (Figure 6.4) in tumour-bearing rats fed the high n-3 diet. The marked increase in splenocyte TNF-α production in tumour-bearing rats fed the high n-3 is consistent with previous reports on lipopolysaccharide-stimulated macrophage production in healthy, fish-oil-fed mice (Chang et al. 1992). Upregulated immune function in tumour-bearing rats fed the high n-3 diet occurred concurrently with the ability of dietary long-chain n-3 fatty acids to maintain n-6 and n-3 fatty acid levels in immune cell phospholipids. However, whether this contributes to the observed diet-induced changes in immune function needs to be further studied. Furthermore, while the length of time that diets were fed in the present study (17 d post-implantation) was sufficient to alter immune cell membrane composition, increase activated CD8$^+$ T cells and enhance splenocyte IFN-$\gamma$ and TNF-$\alpha$ production, it was perhaps not long enough for these mechanisms to impact on tumour growth. R3230AC mammary tumour growth was 31% lower in rats fed long-chain n-3 fatty acids, but this effect was not statistically significant in the present study. However, our results do not preclude benefits of long-chain n-3 fatty acids on later cancer stages, such as improved response to chemotherapy (de Salis and Meckling-Gill 1995), reduction of tumour metastasis (Rose et al. 1995), or prevention of cancer cachexia (Barber et al. 1999), or on earlier stages, such as cancer prevention (Noguchi et al. 1997).

Overall, our results suggest that feeding a physiological level long-chain n-3 fatty acids in a low P/S diet may have beneficial effects on several host immune defenses, including activation of CD8$^+$ T cells and type-1 cytokine (IFN-$\gamma$ and TNF-$\alpha$) production. These immune benefits occur simultaneously with specific changes in the major membrane phospholipids PC and PE in high n-3-fed rats. As membrane composition plays a critical role in immune function, further work is needed to determine the relationship between diet-induced alterations in the phospholipid composition of immune cells during cancer and subsequent upregulation of host defense.

**TABLE 6.1 Fatty Acid Composition of Low n-3 and High n-3 Diets Fed to Rats[1]**

| Fatty Acid | Low n-3 Diet | High n-3 Diet |
|---|---|---|
| | % w/w | |
| C14:0 | 2.2 | 2.8 |
| C16:0 | 21.1 | 21.0 |
| C18:0 | 44.4 | 42.6 |
| C18:1 (9) | 4.6 | 4.5 |
| C18:2 (6) | 23.4 | 18.8 |
| C18:3 (3) | 1.1 | 0.2 |
| C20:5 (3) | nd | 3.2 |
| C22:5 (3) | nd | 0.2 |
| C22:6 (3) | nd | 0.8 |
| SFA | 70.2 | 69.0 |
| MUFA | 4.8 | 6.6 |
| PUFA | 24.5 | 24.0 |
| n-6 PUFA | 23.4 | 19.1 |
| n-3 PUFA | 1.1 | 5.0 |
| n-6/n-3 ratio | 21 | 3.8 |
| P/S ratio | 0.35 | 0.35 |

[1] Values are g/100 g of total fat, except for ratios. Diets contained 200 g/kg of fat from a mixture of sources [safflower oil, hard beef tallow, linseed oil (low n-3 diets only), and fish oil (high n-3 diets only]. Minor fatty acids are not reported, therefore totals do not add up to 100%. Abbreviations used: nd, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; PUFA, sum of polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids.

**TABLE 6.2 Monoclonal Antibodies Used in Indirect Immunofluorescence (Phenotype) Assay**

| Clone[1] | Specificity | Description |
|---|---|---|
| OX19 | CD5 | Reacts with 69 kDa surface glycoprotein on thymocytes and peripheral T cells |
| w3/25 | CD4 | Reacts with 48-53 kDa surface glycoprotein on T helper cells and peritoneal macrophages |
| OX8 | CD8a | Reacts with 34 kDa α chain of CD8 antigen on thymocytes, T suppressor/cytotoxic cells, natural killer (NK) cells, and some activated T helper cells |
| OX12 | Ig κ chain | Reacts with immunoglobulin (Ig) κ chain on B cells |
| OX42 | CD11b/c | Reacts with 160, 103, and 95 kDa cell surface proteins on most resident peritoneal and activated macrophages, granulocytes, monocytes, and dendritic cells |
| 3.2.3 | CD161 | Reacts with NKR-P1A on NK cells and subset of T cells |
| JJ319 | CD28 | Reacts with a costimulatory receptor for activation on T cells and a subset of NK cells |
| OX39 | CD25 | Reacts with the α-chain of the interleukin-2 receptor on activated T and B cells |

[1] All monoclonal antibodies were kindly provided by Dr. A. Rabinovitch (University of Alberta, Edmonton, AB, Canada) with the exceptions of 3.2.3 and OX39 (Cedarlane Laboratories Ltd., Hornby, ON, Canada) and JJ319 (PharMingen, Mississauga, ON, Canada). All mAb were mouse anti-rat IgG.

TABLE 6.3 Effect of Diet and R3230AC Mammary Tumour on [methyl-$^3$H]-Thymidine Incorporation by Splenocytes[1]

| | Healthy | | Tumour-bearing | | Significance, $p \leq$[2] | |
|---|---|---|---|---|---|---|
| **Incubation Time and Mitogen** | **Low n-3 Diet** | **High n-3 Diet** | **Low n-3 Diet** | **High n-3 Diet** | Diet | Tumour |
| | [methyl $^3$H]-thymidine incorporation (Bq/min/2.5 x10$^6$ cells) | | | | | |
| **66 h** | | | | | | |
| Unstimulated | 2745 ± 553 | 2125 ± 288 | 1967 ± 630 | 2886 ± 612 | NS | NS |
| + Con A | 268386 ± 34664 | 234948 ± 34241 | 133650 ± 45300 | 145099 ± 41290 | NS | 0.007 |
| + PMA + Iono | 216542 ± 28409 | 186171 ± 25032 | 109134 ± 36183 | 125649 ± 39807 | NS | 0.01 |
| **78 h** | | | | | | |
| Unstimulated | 1877 ± 503 | 1616 ± 369 | 1749 ± 570 | 2478 ± 607 | NS | NS |
| + Con A | 295525 ± 28003 | 300339 ± 31271 | 169249 ± 50391 | 204260 ± 48423 | NS | 0.007 |
| + PMA + Iono | 161032 ± 21527 | 157622 ± 20968 | 63584 ± 11970 | 101152 ± 30180 | NS | 0.007 |

[1] Values are means ± SEM for healthy and tumour-bearing rats fed a low P/S diet either unsupplemented (Low n-3 Diet) or supplemented with long-chain n-3 fatty acids (High n-3 Diet) ($n \geq 6$/group).

[2] Data were analyzed by two-way ANOVA. No significant interactions were found. NS = $p > 0.05$.

**TABLE 6.4 Effect of Diet and R3230AC Mammary Tumour on Immune Cell Phenotypes in Rat Spleen After Splenocytes Were Cultured for 48 h Without Mitogen[1]**

| | Healthy | | Tumour-bearing | | Significance, $p \leq$[2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenotype** | **Low n-3 Diet** | **High n-3 Diet** | **Low n-3 Diet** | **High n-3 Diet** | Diet | Tumour |
| | | | % of total immune cells in spleen | | | |
| $CD5^+$ T cells | 32 ± 2 | 35 ± 2 | 49 ± 6 | 56 ± 6 | NS | 0.0001 |
| $CD4^+$ T helper cells | 23 ± 1 | 25 ± 2 | 37 ± 5 | 43 ± 5 | NS | 0.0001 |
| $CD8^+$ T suppressor cytotoxic cells | $10 \pm 0^{bc}$ | $8.4 \pm 1.0^{c}$ | $12 \pm 1^{ab}$ | $15 \pm 2^{a}$ | NS | 0.001 |
| B cells | 48 ± 2 | 42 ± 6 | 36 ± 4 | 28 ± 5 | NS | 0.001 |
| Macrophages | 1.8 ± 0.4 | 2.2 ± 0.6 | 4.7 ± 1.4 | 3.0 ± 0.7 | NS | NS |
| CD4/CD8 | 2.3 ± 0.2 | 2.6 ± 0.3 | 3.1 ± 0.4 | 3.0 ± 0.2 | NS | NS |
| $CD28^+$ cells | $43 \pm 2^{bc}$ | $39 \pm 3^{c}$ | $55 \pm 6^{ab}$ | $59 \pm 5^{a}$ | NS | 0.001 |
| $CD25^+$ cells | 1.6 ± 0.4 | 0.9 ± 0.3 | 2.2 ± 0.3 | 2.1 ± 0.7 | NS | 0.03 |

[1] Values are means ± SEM for healthy and tumour-bearing rats fed a low P/S diet either unsupplemented (Low n-3 Diet) or supplemented with long-chain n-3 fatty acids (High n-3 Diet) ($n \geq 6$/group).

[2] Data were analyzed by two-way ANOVA. When a significant interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test. NS = $p > 0.05$.

**TABLE 6.5 Immune Cell Phenotypes in Splenocytes From Healthy and Tumour-Bearing Rats After Splenocytes Were Cultured for 48 h Without Mitogen**[1]

| Immune Cell Phenotype | Healthy | Tumour-bearing | Significance, $p \leq$[2] |
|---|---|---|---|
| | % of total immune cells in spleen | | |
| $CD5^+$ T cells | 33 ± 1 | 52 ± 4 | 0.0001 |
| $CD4^+$ T helper cells | 24 ± 1 | 40 ± 4 | 0.0001 |
| $CD8^+$ T suppressor cytotoxic cells | 9 ± 1 | 13 ± 1 | 0.002 |
| B cells | 45 ± 3 | 33 ± 3 | 0.01 |
| Macrophages | 1.7 ± 0.3 | 3.3 ± 0.7 | NS |
| CD4/CD8 | 2.4 ± 0.2 | 3.1 ± 0.3 | 0.02 |
| $CD28^+$ cells | 41 ± 2 | 57 ± 4 | 0.001 |
| $CD25^+$ cells | 1.3 ± 0.2 | 2.2 ± 0.3 | 0.007 |

[1] Values are means ± SEM Diet did not significantly affect immune cell phenotypes; therefore, rats in the low and high n-3 diet groups within either the healthy or tumour-bearing group were combined ($n \geq 10$/group).

[2] Data were analyzed by Student's *t*-test, NS = $p > 0.05$.

**TABLE 6.6 Effect of Diet and R3230AC Mammary Tumour on Immune Cell Phenotypes in Rat Spleen After Splenocytes Were Stimulated With Phorbol Myrisate Acetate Plus Ionomycin**[1]

| | Healthy | | Tumour-bearing | | Significance, $p \leq$[2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenotype** | **Low n-3 Diet** | **High n-3 Diet** | **Low n-3 Diet** | **High n-3 Diet** | Diet | Tumour |
| | % of total immune cells in spleen | | | | | |
| $CD5^+$ T cells | 26 ± 2 | 26 ± 2 | 25 ± 2 | 27 ± 2 | NS | NS |
| $CD4^+$ T helper cells | 16 ± 1 | 14 ± 2 | 11 ± 1 | 11 ± 1 | NS | 0.02 |
| $CD8^+$ T suppressor cytotoxic cells | $9.8 \pm 1.1^b$ | $8.6 \pm 1.2^b$ | $8.8 \pm 0.8^b$ | $14 \pm 2^a$ | NS | NS |
| B cells | 48 ± 4 | 47 ± 4 | 31 ± 4 | 28 ± 1 | NS | 0.0008 |
| Macrophages | 3.0 ± 0.4 | 3.3 ± 0.3 | 3.2 ± 0.5 | 3.5 ± 0.7 | NS | NS |
| $CD28^+$ cells | 31 ± 3 | 23 ± 4 | 27 ± 2 | 29 ± 2 | NS | NS |
| CD4/CD8 | $1.6 \pm 0.1^a$ | $1.7 \pm 0.2^a$ | $1.3 \pm 0.2^a$ | $0.8 \pm 0.1^b$ | NS | 0.001 |

[1] Values are means ± SEM for healthy and tumour-bearing rats fed a low P/S diet either unsupplemented (Low n-3 Diet) or supplemented with long-chain n-3 fatty acids (High n-3 Diet) ($n \geq 6$/group).

[2] Data were analyzed by two-way ANOVA. When a significant interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test. NS = $p > 0.05$.

**TABLE 6.7 Interleukin-2 Receptor (CD25) Expression in Splenocytes From Healthy and Tumour-Bearing Rats After Splenocyte Stimulation With Phorbol Myrisate Acetate Plus Ionomycin**[1]

| | Healthy | | Tumour-bearing | | Significance, $p \leq$[2] | |
|---|---|---|---|---|---|---|
| **Immune Cell Phenotype** | **Low n-3 Diet** | **High n-3 Diet** | **Low n-3 Diet** | **High n-3 Diet** | Diet | Tumour |
| | % of total immune cells in spleen | | | | | |
| $CD25^+$ $CD5^+$ T cells | 32 ± 2 | 28 ± 2 | 27 ± 2 | 28 ± 0 | NS | NS |
| $CD25^+$ $CD4^+$ T helper cells | 21 ± 1 | 19 ± 1 | 14 ± 1 | 14 ± 1 | NS | 0.0001 |
| $CD25^+$ $CD8^+$ T suppressor/cytotoxic cells | $15 \pm 1^{ab}$ | $13 \pm 1^{b}$ | $12 \pm 1^{b}$ | $17 \pm 2^{a}$ | NS | NS |
| $CD25^+$ B cells | 50 ± 4 | 49 ± 4 | 32 ± 4 | 28 ± 4 | NS | 0.0001 |
| $CD25^+$ Macrophages | 8.5 ± 0.8 | 7.6 ± 0.3 | 6.2 ± 0.6 | 7.0 ± 0.6 | NS | 0.04 |
| $CD25^+$ $CD28^+$ cells | 30 ± 3 | 30 ± 3 | 22 ± 3 | 25 ± 2 | NS | 0.04 |

[1] Values are means ± SEM for healthy and tumour-bearing rats fed a low P/S diet either unsupplemented (Low n-3 Diet) or supplemented with long-chain n-3 fatty acids (High n-3 Diet) ($n \geq 5$/group).

[2] Data were analyzed by two-way ANOVA. When a significant interaction was found, values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test. NS = $p > 0.05$.

TABLE 6.8 Effect of Diet and R3230AC Mammary Tumour on Splenocyte Interferon-γ and Tumour Necrosis Factor-α Production[1]

| | Healthy | | Tumour-bearing | | Significance, $p \leq$[2] | |
|---|---|---|---|---|---|---|
| | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Tumour |
| **Unstimulated** | pg/mL ($10^6$ cells/mL) | | | | | |
| IFN-γ | nd | nd | nd | nd | -- | -- |
| TNF-α | 20 ± 4 | 26 ± 10 | 26 ± 4 | 35 ± 7 | NS | NS |
| **Concanavalin A** | | | | | | |
| IFN-γ | 843 ± 40 | 840 ± 83 | 952 ± 63 | 1034 ± 69 | NS | 0.04 |
| TNF-α | 76 ± 12 | 74 ± 14 | 113 ± 9 | 99 ± 9 | NS | 0.02 |

[1] Values are means ± SEM ($n \geq 5$/group). Splenocytes were isolated from healthy and tumour-bearing rats fed a low P/S diet either unsupplemented (Low n-3 Diet) or supplemented with long-chain n-3 fatty acids (High n-3 Diet). Splenocytes were cultured for 48 h without mitogen (unstimulated) or with Concanavalin A (5 mg/L) and cytokine production was determined by enzyme-linked immunosorbent assay as described in Materials and Methods.

[2] Data were analyzed by two-way ANOVA. No significant interactions were found. NS = $p > 0.05$.

**TABLE 6.9 Effect of Diet and Tumour on Fatty Acid Composition of Phosphatidylcholine in Freshly Isolated Splenocytes**[1]

| | Healthy Rats | | Tumour-bearing Rats | | Significance, p<[2] | |
|---|---|---|---|---|---|---|
| | **Low n-3 Diet** | **High n-3 Diet** | **Low n-3 Diet** | **High n-3 Diet** | **Diet** | **Tumour** |
| **Fatty Acid** | % (w/w) of total fatty acids | | | | | |
| C14:0 | 1.2 ± 0.7 | 0.93 ± 0.22 | 1.4 ± 0.5 | 0.53 ± 0.14 | NS | NS |
| C16:0 | 37 ± 2 | 40 ± 1 | 39 ± 1 | 38 ± 1 | NS | NS |
| C16:1(7) | 0.70 ± 0.27 | 0.68 ± 0.12 | 0.54 ± 0.05 | 0.71 ± 0.12 | NS | NS |
| C18:0 | 17 ± 1 | 16 ± 1 | 15 ± 1 | 14 ± 1 | NS | NS |
| C18:1(9) | 8.3 ± 0.6 | 7.9 ± 0.2 | 7.7 ± 0.6 | 8.6 ± 0.5 | NS | NS |
| C18:1(7) | 3.0 ± 0.3 | 3.0 ± 0.3 | 2.8 ± 0.2 | 3.1 ± 0.1 | NS | NS |
| C18:2(6) | 11 ± 2$^{b}$ | 16 ± 0.5$^{a}$ | 11 ± 1$^{b}$ | 16 ± 1$^{a}$ | 0.001 | NS |
| C18:3(3) | 0.09 ± 0.02 | 0.12 ± 0.03 | 0.14 ± 0.02 | 0.10 ± 0.01 | NS | NS |
| C20:0 | 0.21 ± 0.12 | 0.27 ± 0.11 | 0.12 ± 0.03 | 0.07 ± 0.02 | NS | NS |
| C20:1(9) | 0.70 ± 0.18 | 0.59 ± 0.08 | 0.48 ± 0.10 | 0.67 ± 0.11 | NS | NS |
| C20:1(7) | 0.40 ± 0.09 | 0.37 ± 0.09 | 0.28 ± 0.04 | 0.23 ± 0.07 | NS | NS |
| C20:2(6) | 1.4 ± 0.1$^{a}$ | 0.85 ± 0.14$^{b}$ | 1.3 ± 0.1$^{a}$ | 1.2 ± 0.1$^{a}$ | 0.01 | NS |
| C20:3(9) | 0.22 ± 0.11 | 0.05 ± 0.01 | 0.07 ± 0.02 | 0.05 ± 0.01 | NS | NS |
| C20:3(6) | 0.98 ± 0.10 | 0.74 ± 0.13 | 0.93 ± 0.05 | 0.94 ± 0.04 | NS | NS |
| C20:4(6) | 13 ± 2$^{ab}$ | 9.0 ± 0.8$^{c}$ | 16 ± 1$^{a}$ | 11 ± 1$^{bc}$ | 0.002 | NS |
| C20:5(3) | 0.51 ± 0.18$^{b}$ | 1.1 ± 0.1$^{a}$ | 0.04 ± 0.01$^{c}$ | 1.1 ± 0.2$^{a}$ | 0.001 | NS |
| C22:4(6) | 0.80 ± 0.13$^{a}$ | 0.26 ± 0.01$^{b}$ | 0.88 ± 0.09$^{a}$ | 0.42 ± 0.12$^{b}$ | 0.001 | NS |
| C22:5(6) | 0.09 ± 0.04$^{ab}$ | nd$^{b}$ | 0.18 ± 0.05$^{a}$ | 0.05 ± 0.02$^{b}$ | 0.001 | NS |
| C22:5(3) | 0.91 ± 0.25$^{bc}$ | 1.7 ± 0.2$^{a}$ | 0.50 ± 0.10$^{c}$ | 1.3 ± 0.2$^{ab}$ | 0.001 | NS |
| C22:6(3) | 0.99 ± 0.13$^{ab}$ | 1.2 ± 0.1$^{a}$ | 0.71 ± 0.04$^{b}$ | 1.2 ± 0.1$^{a}$ | 0.002 | NS |
| SFA | 57 ± 3 | 54 ± 1 | 57 ± 2 | 53 ± 1 | NS | NS |
| MUFA | 13 ± 1 | 13 ± 0.2 | 12 ± 1 | 14 ± 1 | NS | NS |
| P/S ratio | 0.55 ± 0.07 | 0.55 ± 0.01 | 0.56 ± 0.05 | 0.63 ± 0.03 | NS | NS |
| n-6 PUFA | 27 ± 3 | 26 ± 0.3 | 30 ± 2 | 30 ± 1 | NS | NS |
| n-3 PUFA | 2.5 ± 0.5$^{b}$ | 4.4 ± 0.3$^{a}$ | 1.4 ± 0.1$^{c}$ | 4.1 ± 0.3$^{a}$ | 0.001 | 0.05 |
| n-6/n-3 | 13 ± 2$^{b}$ | 6.4 ± 0.2$^{c}$ | 22 ± 3$^{a}$ | 7.2 ± 0.3$^{bc}$ | 0.001 | 0.02 |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 6$ per diet). Abbreviations used: nd, not dectectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; n-6 PUFA, sum of n-6 polyunsaturated fatty acids; n-3 PUFA, sum of n-3 polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids.

[2] Significant effect of diet and tumour as determined by two-way ANOVA, NS = $p > 0.05$. Values within a row without a common superscript are significantly different ($p \leq 0.05$).

**TABLE 6.10 Effect of Diet and Tumour on Fatty Acid Composition of Phosphatidylethanolamine in Freshly Isolated Splenocytes**[1]

| | Healthy Rats | | Tumour-bearing Rats | | Significance, p<[2] | |
|---|---|---|---|---|---|---|
| | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Tumour |
| **Fatty Acid** | % (w/w) of total fatty acids | | | | | |
| C14:0 | 2.4 ± 0.5 | 2.5 ± 0.4 | 2.6 ± 0.5 | 1.3 ± 0.4 | NS | NS |
| C16:0 | 12 ± 1 | 10 ± 1 | 11 ± 1 | 9.5 ± 0.4 | NS | NS |
| C16:1(7) | 0.38 ± 0.08 | 0.60 ± 0.07 | 0.62 ± 0.17 | 0.67 ± 0.18 | NS | NS |
| C18:0 | 21 ± 1 | 20 ± 0.4 | 19 ± 0.4 | 20 ± 1 | NS | NS |
| C18:1(9) | 16 ± 2 | 13 ± 1 | 14 ± 2 | 12 ± 2 | NS | NS |
| C18:1(7) | 1.6 ± 0.2 | 1.6 ± 0.1 | 1.4 ± 0.1 | 1.5 ± 0.1 | NS | NS |
| C18:2(6) | 6.6 ± 0.5$^{b}$ | 8.3 ± 0.2$^{a}$ | 6.7 ± 0.2$^{b}$ | 8.8 ± 0.1$^{a}$ | 0.001 | NS |
| C18:3(3) | 0.54 ± 0.09 | 0.48 ± 0.07 | 0.56 ± 0.10 | 0.50 ± 0.09 | NS | NS |
| C20:1(9) | 0.11 ± 0.04 | 0.12 ± 0.03 | 0.13 ± 0.03 | 0.17 ± 0.02 | NS | NS |
| C20:1(7) | 0.32 ± 0.02 | 0.22 ± 0.04 | 0.21 ± 0.05 | 0.12 ± 0.05 | NS | NS |
| C20:2(6) | 0.37 ± 0.03 | 0.33 ± 0.01 | 0.34 ± 0.04 | 0.35 ± 0.02 | NS | NS |
| C20:3(9) | 0.14 ± 0.04 | 0.13 ± 0.02 | 0.14 ± 0.03 | 0.13 ± 0.01 | NS | NS |
| C20:3(6) | 0.66 ± 0.07 | 0.73 ± 0.03 | 0.65 ± 0.06 | 0.75 ± 0.04 | NS | NS |
| C20:4(6) | 27 ± 2$^{a}$ | 21 ± 1$^{b}$ | 27 ± 2$^{a}$ | 22 ± 1$^{ab}$ | 0.01 | NS |
| C20:5(3) | 0.13 ± 0.02$^{b}$ | 3.4 ± 0.3$^{a}$ | 0.14 ± 0.04$^{b}$ | 3.5 ± 0.3$^{a}$ | 0.001 | NS |
| C22:4(6) | 3.9 ± 0.4$^{a}$ | 0.88 ± 0.11$^{b}$ | 4.2 ± 0.4$^{a}$ | 0.94 ± 0.03$^{b}$ | 0.001 | NS |
| C22:5(6) | 0.76 ± 0.07$^{b}$ | 0.28 ± 0.07$^{c}$ | 1.1 ± 0.1$^{a}$ | 0.10 ± 0.02$^{c}$ | 0.001 | NS |
| C22:5(3) | 1.2 ± 0.2$^{b}$ | 5.4 ± 0.6$^{a}$ | 1.1 ± 0.1$^{b}$ | 5.2 ± 0.3$^{a}$ | 0.001 | NS |
| C22:6(3) | 2.5 ± 0.4$^{b}$ | 4.8 ± 0.5$^{a}$ | 2.1 ± 0.2$^{b}$ | 4.8 ± 0.2$^{a}$ | 0.001 | NS |
| SFA | 38 ± 1 | 38 ± 1 | 39 ± 1 | 38 ± 1 | NS | NS |
| MUFA | 19 ± 3 | 16 ± 1 | 17 ± 2 | 15 ± 2 | NS | NS |
| P/S ratio | 1.1 ± 0.1 | 1.2 ± 0.1 | 1.1 ± 0.1 | 1.2 ± 0.1 | NS | NS |
| n-6 PUFA | 38 ± 3$^{ab}$ | 32 ± 1$^{c}$ | 40 ± 2$^{a}$ | 33 ± 1$^{bc}$ | 0.005 | NS |
| n-3 PUFA | 4.6 ± 0.5$^{b}$ | 14 ± 1$^{a}$ | 4.0 ± 0.2$^{b}$ | 14 ± 1$^{a}$ | 0.001 | NS |
| n-6/n-3 | 8.9 ± 0.6$^{a}$ | 2.3 ± 0.2$^{b}$ | 10 ± 0.4$^{a}$ | 2.4 ± 0.1$^{b}$ | 0.001 | NS |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 5$ per diet). Abbreviations used are defined in the legend to Table 6.9.

[2] Significant effect of diet and tumour as determined by two-way ANOVA, NS = $p > 0.05$. Values within a row without a common superscript are significantly different ($p \leq 0.05$).

**TABLE 6.11 Effect of Diet and Tumour on Fatty Acid Composition of Phosphatidylcholine in Splenocytes Stimulated with Concanavalin A[1]**

| | Healthy Rats | | Tumour-bearing Rats | | Significance, p<[2] | |
|---|---|---|---|---|---|---|
| | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Tumour |
| **Fatty Acid** | % (w/w) of total fatty acids | | | | | |
| C14:0 | 1.9 ± 0.5 | 0.74 ± 0.31 | 1.9 ± 0.6 | 1.1 ± 0.2 | NS | NS |
| C16:0 | 43 ± 2 | 39 ± 1 | 40 ± 1 | 38 ± 2 | NS | NS |
| C16:1(7) | 0.28 ± 0.06 | 0.44 ± 0.10 | 0.44 ± 0.21 | 0.25 ± 0.08 | NS | NS |
| C18:0 | 21 ± 2 | 20 ± 2 | 20 ± 1 | 22 ± 1 | NS | NS |
| C18:1(9) | 7.1 ± 0.7$^{b}$ | 11 ± 1$^{a}$ | 10 ± 1$^{a}$ | 9.3 ± 0.6$^{ab}$ | NS | NS |
| C18:1(7) | 3.1 ± 0.4 | 3.9 ± 0.4 | 3.2 ± 0.3 | 2.7 ± 0.4 | NS | NS |
| C18:2(6) | 7.6 ± 1.8$^{bc}$ | 12 ± 1$^{a}$ | 7.0 ± 0.6$^{c}$ | 11 ± 1$^{ab}$ | 0.01 | NS |
| C18:3(3) | 0.10 ± 0.04 | 0.16 ± 0.08 | 0.31 ± 0.11 | nd | NS | NS |
| C20:0 | 0.17 ± 0.07 | nd | 0.33 ± 0.22 | nd | NS | NS |
| C20:1(9) | 0.52 ± 0.13 | 0.64 ± 0.06 | 0.54 ± 0.15 | 0.55 ± 0.21 | NS | NS |
| C20:1(7) | 0.63 ± 0.34 | 0.96 ± 0.21 | 0.22 ± 0.05 | 0.39 ± 0.12 | NS | NS |
| C20:2(6) | 0.84 ± 0.14$^{a}$ | 1.0 ± 0.3$^{a}$ | 0.71 ± 0.15$^{a}$ | 0.07 ± 0.03$^{b}$ | NS | 0.01 |
| C20:3(9) | nd$^{b}$ | 0.14 ± 0.07$^{b}$ | 0.29 ± 0.10$^{ab}$ | 0.59 ± 0.18$^{a}$ | NS | 0.003 |
| C20:3(6) | 0.60 ± 0.17 | 0.99 ± 0.38 | 0.61 ± 0.16 | 0.76 ± 0.26 | NS | NS |
| C20:4(6) | 10 ± 2 | 14 ± 2 | 10 ± 1 | 10 ± 1 | NS | NS |
| C20:5(3) | 0.22 ± 0.10 | 0.33 ± 0.06 | 0.31 ± 0.09 | 0.49 ± 0.09 | NS | NS |
| C22:4(6) | 0.77 ± 0.13$^{ab}$ | 0.63 ± 0.12$^{b}$ | 1.2 ± 0.2$^{a}$ | 0.35 ± 0.11$^{b}$ | 0.01 | NS |
| C22:5(6) | 0.08 ± 0.04 | 0.10 ± 0.07 | nd | nd | NS | NS |
| C22:5(3) | 1.3 ± 0.6 | 1.4 ± 0.2 | 0.53 ± 0.12 | 1.1 ± 0.5 | NS | NS |
| C22:6(3) | 0.87 ± 0.13$^{b}$ | 1.4 ± 0.2$^{a}$ | 0.71 ± 0.12$^{b}$ | 1.1 ± 0.1$^{ab}$ | 0.01 | NS |
| SFA | 67 ± 4 | 55 ± 4 | 63 ± 2 | 62 ± 3 | NS | NS |
| MUFA | 12 ± 1$^{b}$ | 17 ± 1$^{a}$ | 15 ± 2$^{ab}$ | 14 ± 1$^{ab}$ | NS | NS |
| P/S ratio | 0.34 ± 0.08 | 0.54 ± 0.11 | 0.35 ± 0.04 | 0.41 ± 0.08 | NS | NS |
| n-6 PUFA | 18 ± 4 | 24 ± 4 | 19 ± 2 | 21 ± 3 | NS | NS |
| n-3 PUFA | 1.9 ± 0.2$^{b}$ | 3.6 ± 0.6$^{a}$ | 2.3 ± 0.4$^{ab}$ | 2.7 ± 0.6$^{ab}$ | 0.04 | NS |
| n-6/n-3 | 11 ± 4 | 5.6 ± 0.8 | 8.3 ± 1.6 | 9.1 ± 2.4 | NS | NS |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 6$ per diet). Abbreviations used are defined in the legend to Table 6.9.

[2] Significant effect of diet and tumour as determined by two-way ANOVA, NS = $p > 0.05$. Values within a row without a common superscript are significantly different ($p \leq 0.05$).

**TABLE 6.12 Effect of Diet and Tumour on Fatty Acid Composition of Phosphatidylethanolamine in Splenocytes Stimulated with Concanavalin A**[1]

| | Healthy Rats | | Tumour-bearing Rats | | Significance, p<[2] | |
|---|---|---|---|---|---|---|
| | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Tumour |
| **Fatty Acid** | % (w/w) of total fatty acids | | | | | |
| C14:0 | 4.7 ± 0.9 | 4.3 ± 0.4 | 4.7 ± 0.8 | 3.8 ± 1.0 | NS | NS |
| C16:0 | 25 ± 6 | 22 ± 3 | 20 ± 1 | 24 ± 3 | NS | NS |
| C16:1(7) | 1.2 ± 0.6 | 1.0 ± 0.3 | 0.57 ± 0.22 | 0.29 ± 0.12 | NS | NS |
| C18:0 | 18 ± 2 | 19 ± 2 | 21 ± 3 | 23 ± 4 | NS | NS |
| C18:1(9) | 16 ± 4 | 21 ± 5 | 19 ± 7 | 14 ± 7 | NS | NS |
| C18:1(7) | 0.78 ± 0.29 | 2.2 ± 0.6 | 1.0 ± 0.2 | 1.1 ± 0.3 | NS | NS |
| C18:2(6) | 5.1 ± 1.1 | 6.9 ± 1.8 | 6.6 ± 1.7 | 5.5 ± 1.6 | NS | NS |
| C18:3(3) | 0.50 ± 0.15 | 0.34 ± 0.07 | 0.66 ± 0.22 | 0.78 ± 0.20 | NS | NS |
| C20:0 | 0.33 ± 0.17 | 0.96 ± 0.46 | 0.26 ± 0.12 | 0.41 ± 0.24 | NS | NS |
| C20:1(9) | 0.39 ± 0.15 | 0.20 ± 0.11 | 0.51 ± 0.24 | 0.53 ± 0.19 | NS | NS |
| C20:1(7) | 0.58 ± 0.26 | 1.1 ± 0.5 | 0.28 ± 0.09 | 0.47 ± 0.23 | NS | NS |
| C20:2(6) | 1.1 ± 0.9$^{ab}$ | 0.21 ± 0.08$^{b}$ | 0.64 ± 0.32$^{b}$ | 4.8 ± 1.8$^{a}$ | NS | 0.04 |
| C20:3(9) | 0.12 ± 0.06 | 0.20 ± 0.04 | 0.20 ± 0.12 | 0.16 ± 0.10 | NS | NS |
| C20:3(6) | 0.63 ± 0.21 | 0.39 ± 0.11 | 0.54 ± 0.19 | 0.46 ± 0.21 | NS | NS |
| C20:4(6) | 13 ± 5 | 10 ± 2 | 13 ± 4 | 7.7 ± 3.1 | NS | NS |
| C20:5(3) | 0.07 ± 0.02 | 0.40 ± 0.08 | 0.21 ± 0.10 | 0.63 ± 0.37 | NS | NS |
| C22:4(6) | 2.2 ± 1.0 | 1.0 ± 0.3 | 2.5 ± 0.8 | 0.92 ± 0.29 | NS | NS |
| C22:5(6) | 0.80 ± 0.34 | 0.30 ± 0.09 | 0.47 ± 0.08 | 0.69 ± 0.24 | NS | NS |
| C22:5(3) | 1.3 ± 0.4 | 1.9 ± 0.3 | 0.62 ± 0.20 | 1.5 ± 0.6 | NS | NS |
| C22:6(3) | 3.1 ± 1.4 | 2.3 ± 0.5 | 1.3 ± 0.4 | 1.9 ± 0.5 | NS | NS |
| SFA | 51 ± 6 | 48 ± 5 | 51 ± 4 | 55 ± 7 | NS | NS |
| MUFA | 21 ± 4 | 26 ± 5 | 23 ± 7 | 19 ± 7 | NS | NS |
| P/S ratio | 0.68 ± 0.24 | 0.55 ± 0.05 | 0.52 ± 0.07 | 0.49 ± 0.11 | NS | NS |
| n-6 PUFA | 23 ± 7 | 19 ± 0.5 | 23 ± 3 | 20 ± 3 | NS | NS |
| n-3 PUFA | 5.1 ± 1.6 | 5.4 ± 0.7 | 3.1 ± 0.8 | 5.1 ± 1.5 | NS | NS |
| n-6/n-3 | 5.4 ± 2.0 | 3.5 ± 0.4 | 8.5 ± 1.2 | 4.9 ± 0.9 | NS | NS |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 6$ per diet). Abbreviations used are defined in the legend to Table 6.9.

[2] Significant effect of diet and tumour as determined by two-way ANOVA, NS = $p > 0.05$. Values within a row without a common superscript are significantly different ($p \leq 0.05$).

**TABLE 6.13 Effect of Diet and Tumour on Fatty Acid Composition of Phosphatidylcholine in Splenocytes Stimulated with Phorbol Myristate Acetate Plus Ionomycin[1]**

| | Healthy Rats | | Tumour-bearing Rats | | Significance, p<[2] | |
|---|---|---|---|---|---|---|
| | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Tumour |
| **Fatty Acid** | % (w/w) of total fatty acids | | | | | |
| C14:0 | 0.23 ± 0.10 | 1.2 ± 0.6 | 1.7 ± 0.7 | 2.3 ± 0.9 | NS | NS |
| C16:0 | 31 ± 3 | 33 ± 2 | 37 ± 2 | 36 ± 2 | NS | NS |
| C16:1(7) | 0.15 ± 0.06 | 0.39 ± 0.10 | 0.58 ± 0.45 | 0.71 ± 0.27 | NS | NS |
| C18:0 | 21 ± 1 | 19 ± 1 | 20 ± 1 | 20 ± 2 | NS | NS |
| C18:1(9) | 11 ± 1 | 11 ± 1 | 13 ± 1 | 12 ± 2 | NS | NS |
| C18:1(7) | $2.4 \pm 0.6^{b}$ | $4.0 \pm 0.2^{a}$ | $3.9 \pm 0.3^{a}$ | $3.9 \pm 0.4^{a}$ | 0.04 | NS |
| C18:2(6) | 9.0 ± 0.6 | 12 ± 1 | 8.4 ± 0.8 | 12 ± 2 | NS | NS |
| C18:3(3) | 0.18 ± 0.07 | 0.12 ± 0.05 | 0.15 ± 0.12 | 0.31 ± 0.10 | NS | NS |
| C20:0 | 0.02 ± 0.02 | 0.09 ± 0.05 | 0.10 ± 0.06 | 0.00 ± 0.00 | NS | NS |
| C20:1(9) | 0.70 ± 0.36 | 0.42 ± 0.12 | 0.58 ± 0.12 | 0.61 ± 0.12 | NS | NS |
| C20:1(7) | 0.33 ± 0.07 | 0.19 ± 0.06 | 0.11 ± 0.05 | 0.26 ± 0.12 | NS | NS |
| C20:2(6) | $1.3 \pm 0.4^{a}$ | $0.30 \pm 0.11^{b}$ | $0.51 \pm 0.18^{b}$ | $0.90 \pm 0.30^{ab}$ | NS | NS |
| C20:3(9) | 0.84 ± 0.19 | 0.68 ± 0.32 | 0.72 ± 0.18 | 0.50 ± 0.19 | NS | NS |
| C20:3(6) | $1.8 \pm 0.3^{a}$ | $1.0 \pm 0.3^{ab}$ | $0.43 \pm 0.27^{b}$ | $0.75 \pm 0.33^{b}$ | NS | 0.02 |
| C20:4(6) | 15 ± 3 | 10 ± 1 | 9.0 ± 3.9 | 4.0 ± 2.3 | NS | NS |
| C20:5(3) | 0.19 ± 0.04 | 0.57 ± 0.17 | 0.21 ± 0.02 | 0.36 ± 0.06 | NS | NS |
| C22:4(6) | $1.2 \pm 0.4^{a}$ | $0.57 \pm 0.09^{b}$ | $0.14 \pm 0.06^{b}$ | $0.56 \pm 0.08^{b}$ | NS | 0.01 |
| C22:5(6) | $0.52 \pm 0.24^{a}$ | $0.10 \pm 0.04^{b}$ | $0.31 \pm 0.19^{ab}$ | $0.00 \pm 0.00^{b}$ | 0.01 | NS |
| C22:5(3) | $0.47 \pm 0.28^{b}$ | $1.2 \pm 0.1^{a}$ | $0.40 \pm 0.15^{b}$ | $1.4 \pm 0.3^{a}$ | 0.001 | NS |
| C22:6(3) | 0.67 ± 0.20 | 0.95 ± 0.21 | 0.49 ± 0.31 | 1.1 ± 0.1 | NS | NS |
| SFA | 53 ± 3 | 53 ± 2 | 58 ± 2 | 57 ± 3 | NS | NS |
| MUFA | 16 ± 0.4 | 19 ± 1 | 18 ± 2 | 18 ± 2 | NS | NS |
| P/S ratio | 0.61 ± 0.09 | 0.51 ± 0.06 | 0.38 ± 0.09 | 0.39 ± 0.06 | NS | NS |
| n-6 PUFA | $29 \pm 3^{a}$ | $23 \pm 2^{ab}$ | $19 \pm 4^{b}$ | $18 \pm 3^{b}$ | NS | 0.02 |
| n-3 PUFA | $1.5 \pm 0.3^{b}$ | $3.3 \pm 0.4^{a}$ | $1.6 \pm 0.2^{b}$ | $3.1 \pm 0.6^{a}$ | 0.001 | NS |
| n-6/n-3 | $22 \pm 4^{a}$ | $7.5 \pm 0.8^{b}$ | $12 \pm 3^{b}$ | $6.3 \pm 1.4^{b}$ | 0.001 | 0.02 |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 6$ per diet). Abbreviations used are defined in the legend to Table 6.9.

[2] Significant effect of diet and tumour as determined by two-way ANOVA, NS = $p > 0.05$. Values within a row without a common superscript are significantly different ($p \leq 0.05$).

**TABLE 6.14 Effect of Diet and Tumour on Fatty Acid Composition of Phosphatidylethanolamine in Splenocytes Stimulated with Phorbol Myristate Acetate Plus Ionomycin**[1]

| | Healthy Rats | | Tumour-bearing Rats | | Significance, p<[2] | |
|---|---|---|---|---|---|---|
| | Low n-3 Diet | High n-3 Diet | Low n-3 Diet | High n-3 Diet | Diet | Tumour |
| **Fatty Acid** | % (w/w) of total fatty acids | | | | | |
| C14:0 | 4.4 ± 1.2 | 3.4 ± 0.7 | 4.2 ± 1.0 | 1.4 ± 0.3 | NS | NS |
| C16:0 | 23 ± 3 | 22 ± 2 | 24 ± 3 | 22 ± 4 | NS | NS |
| C16:1(7) | $0.50 \pm 0.08^{ab}$ | $0.54 \pm 0.12^{ab}$ | $0.75 \pm 0.07^{a}$ | $0.32 \pm 0.14^{b}$ | NS | NS |
| C18:0 | $17 \pm 2^{b}$ | $16 \pm 1^{b}$ | $18 \pm 2^{ab}$ | $22 \pm 1^{a}$ | NS | 0.04 |
| C18:1(9) | 23 ± 4 | 27 ± 3 | 21 ± 4 | 15 ± 1 | NS | NS |
| C18:1(7) | 1.4 ± 0.3 | 2.1 ± 0.2 | 1.6 ± 0.4 | 1.7 ± 0.5 | NS | NS |
| C18:2(6) | 7.3 ± 1.0 | 9.0 ± 0.9 | 6.6 ± 1.1 | 7.8 ± 0.8 | NS | NS |
| C18:3(3) | 0.49 ± 0.12 | 0.89 ± 0.11 | 0.61 ± 0.16 | 0.65 ± 0.11 | NS | NS |
| C20:0 | $0.07 \pm 0.06^{b}$ | $0.39 \pm 0.10^{ab}$ | $0.12 \pm 0.08^{b}$ | $1.3 \pm 0.9^{a}$ | 0.05 | NS |
| C20:1(9) | $0.36 \pm 0.15^{a}$ | $\text{nd}^{b}$ | $0.43 \pm 0.17^{a}$ | $\text{nd}^{b}$ | 0.001 | NS |
| C20:1(7) | 0.25 ± 0.12 | 0.31 ± 0.05 | 0.13 ± 0.08 | 0.90 ± 0.60 | NS | NS |
| C20:2(6) | $0.92 \pm 0.36^{a}$ | $0.11 \pm 0.03^{b}$ | $0.71 \pm 0.17^{ab}$ | $0.41 \pm 0.19^{ab}$ | 0.02 | NS |
| C20:3(9) | 0.11 ± 0.06 | 0.20 ± 0.05 | 0.26 ± 0.15 | 0.16 ± 0.08 | NS | NS |
| C20:3(6) | 0.55 ± 0.12 | 0.19 ± 0.05 | 0.61 ± 0.28 | 0.68 ± 0.26 | NS | NS |
| C20:4(6) | 10 ± 4 | 6.4 ± 0.8 | 12 ± 4 | 15 ± 3 | NS | NS |
| C20:5(3) | $0.40 \pm 0.14^{b}$ | $0.65 \pm 0.19^{ab}$ | $0.31 \pm 0.05^{b}$ | $1.5 \pm 0.7^{a}$ | 0.04 | NS |
| C22:4(6) | 2.0 ± 0.5 | 0.59 ± 0.12 | 1.1 ± 0.6 | 1.3 ± 0.2 | NS | NS |
| C22:5(6) | 0.40 ± 0.07 | 0.13 ± 0.05 | 1.2 ± 0.9 | 0.42 ± 0.08 | NS | NS |
| C22:5(3) | $0.88 \pm 0.35^{b}$ | $1.3 \pm 0.2^{b}$ | $0.90 \pm 0.22^{b}$ | $3.1 \pm 0.9^{a}$ | 0.01 | 0.04 |
| C22:6(3) | $1.6 \pm 0.4^{b}$ | $1.4 \pm 0.1^{b}$ | $1.6 \pm 0.3^{b}$ | $3.4 \pm 0.7^{a}$ | 0.02 | 0.01 |
| SFA | 42 ± 3 | 41 ± 3 | 43 ± 3 | 44 ± 4 | NS | NS |
| MUFA | $27 \pm 4^{ab}$ | $33 \pm 3^{a}$ | $25 \pm 4^{ab}$ | $19 \pm 2^{b}$ | NS | 0.04 |
| P/S ratio | 0.63 ± 0.14 | 0.57 ± 0.06 | 0.62 ± 0.14 | 0.84 ± 0.21 | NS | NS |
| n-6 PUFA | 21 ± 4 | 17 ± 1 | 22 ± 5 | 25 ± 3 | NS | NS |
| n-3 PUFA | $2.8 \pm 0.4^{b}$ | $4.6 \pm 0.4^{b}$ | $3.4 \pm 0.3^{b}$ | $9.6 \pm 2.3^{a}$ | 0.001 | 0.01 |
| n-6/n-3 | $7.1 \pm 1.6^{a}$ | $4.0 \pm 0.5^{bc}$ | $6.2 \pm 1.0^{ab}$ | $2.8 \pm 0.3^{c}$ | 0.005 | NS |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 6$ per diet). Abbreviations used are defined in the legend to Table 6.9.

[2] Significant effect of diet and tumour as determined by two-way ANOVA, NS = $p > 0.05$. Values within a row without a common superscript are significantly different ($p \leq 0.05$).

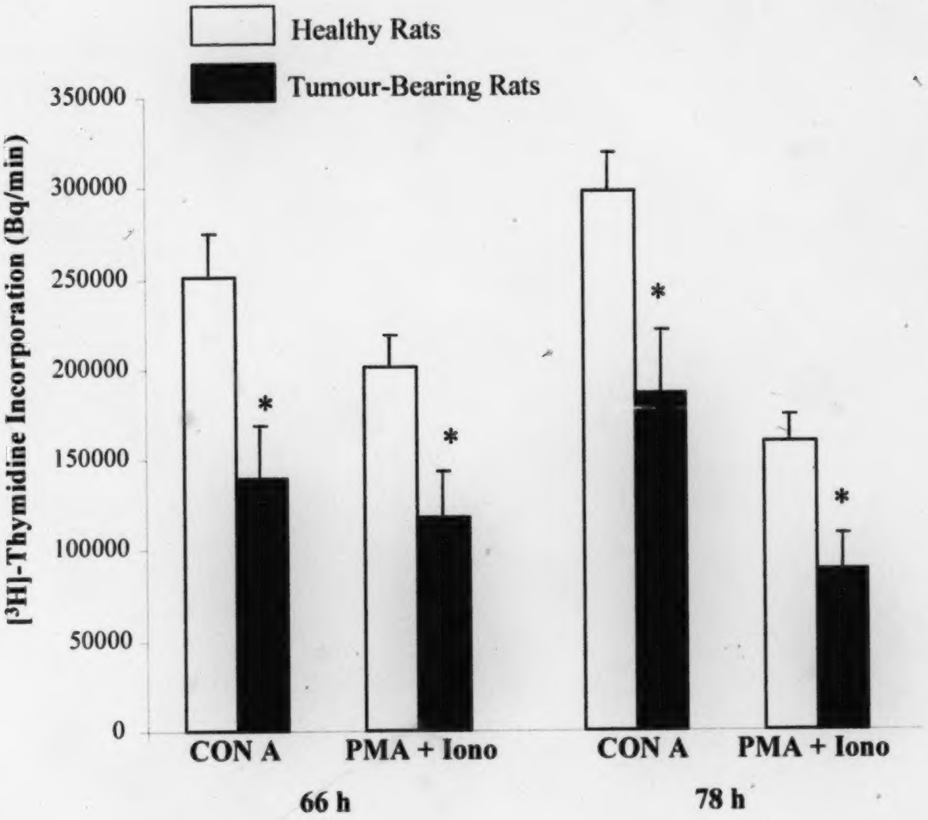

**FIGURE 6.1 Effect of R3230AC Mammary Tumour on Splenocyte [$^3$H]-Thymidine Incorporation in Response to 66 and 78 h Mitogen Incubation.** Splenocytes were cultured for 66 or 78 h with either Concanavalin A (Con A, 5 mg/L) or Phorbol Myrisate Acetate (PMA, 30 µg/L) plus Ionomycin (Iono, 0.75 µmol/L) as described in Materials and Methods. Diet did not significantly affect [$^3$H]-thymidine uptake; therefore, rats in the low and high n-3 diet groups within either the healthy or tumour-bearing group were combined. Bars represent means ± SEM (Healthy rats, *n* = [illegible]9; Tumour-Bearing rats, *n* = 14). For each incubation time and mitogen, * indicates a significant ($p < 0.01$) effect of the tumour as determined by Student's *t*-test.

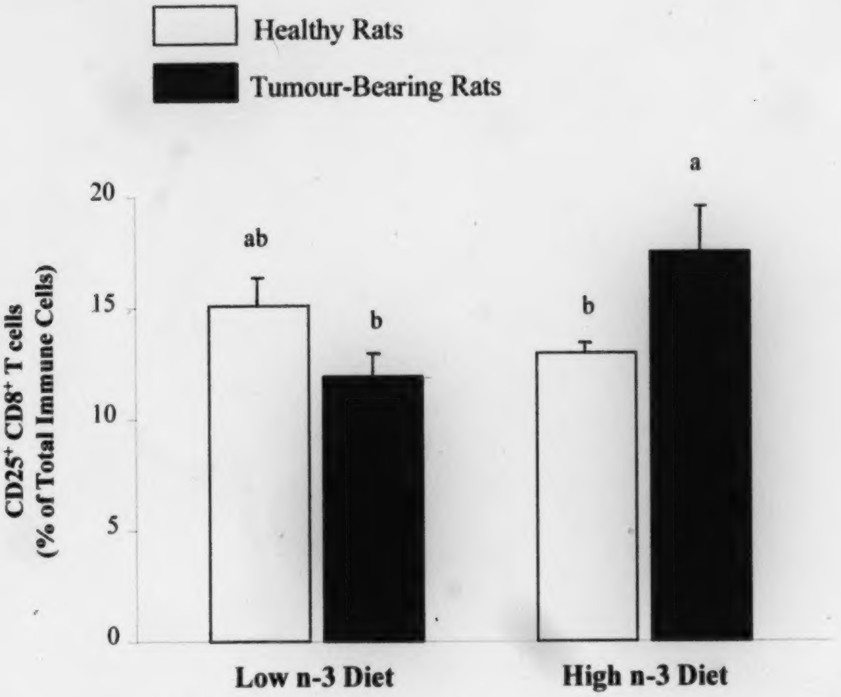

**FIGURE 6.2 Effect of Long-Chain n-3 Fatty Acids and Tumour Burden on the Relative Proportion of $CD25^+$ $CD8^+$ T Cells After Phorbol Myristate Acetate Plus Ionomycin Stimulation.** Splenocytes isolated from healthy and tumour-bearing rats fed either the low or high n-3 diet were stimulated for 48 h with Phorbol Myristate Acetate (PMA, 30 μg/L) plus Ionomycin (0.75 μmol/L). Immune cell phenotypes were identified by indirect immunofluorescence assay and flow cytometry as described in Materials and Methods. Bars represent means ± SEM (Healthy, $n \geq 9$/diet; Tumour-Bearing, $n =$ 7/diet). The effects of diet and tumour were analyzed by two-way ANOVA. Bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

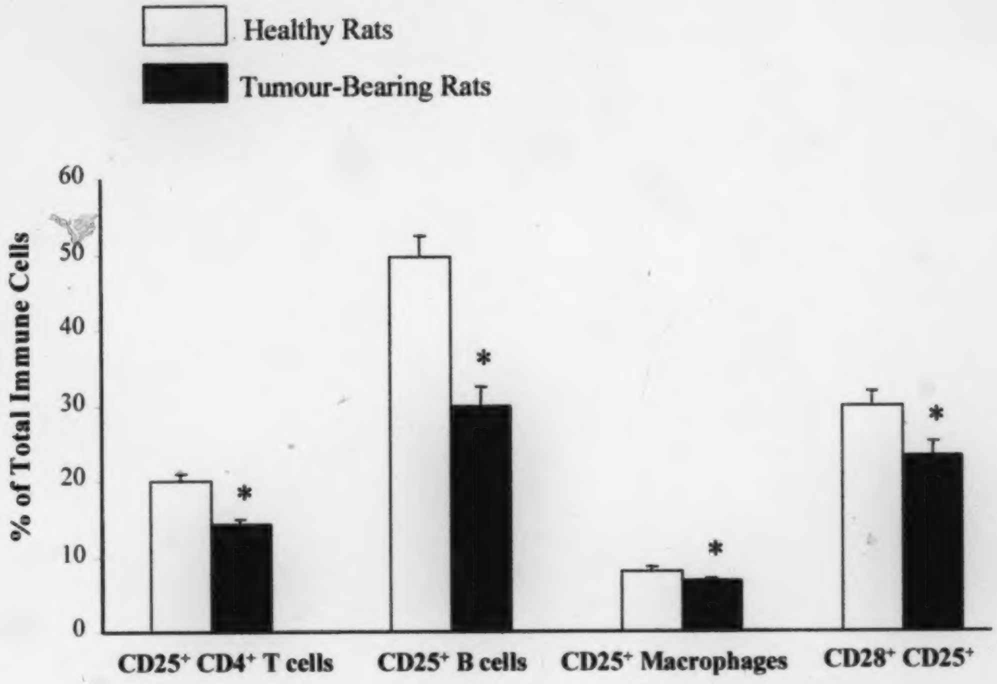

**FIGURE 6.3 Effect of R3230AC Mammary Tumour on Interleukin-2 Receptor (CD25) Expression After Phorbol Myrsisate Acetate Plus Ionomycin Stimulation.** Isolated splenocytes were stimulated for 48 h with Phorbol Myristate Acetate (PMA, 30 μg/L) plus Ionomycin (0.75 μmol/L) and immune cell phenotypes were identified by indirect immunofluorescence assay and flow cytometry as described in Materials and Methods. Diet did not significantly affect CD25 expression; therefore, rats in the low and high n-3 diet groups within either the healthy or tumour-bearing group were combined. Bars represent means ± SEM (Healthy, *n* =19; Tumour-Bearing, *n* = 14). For each immune cell phenotype, * indicates a significant ($p < 0.05$) effect of tumour burden as determined by Student's *t*-test.

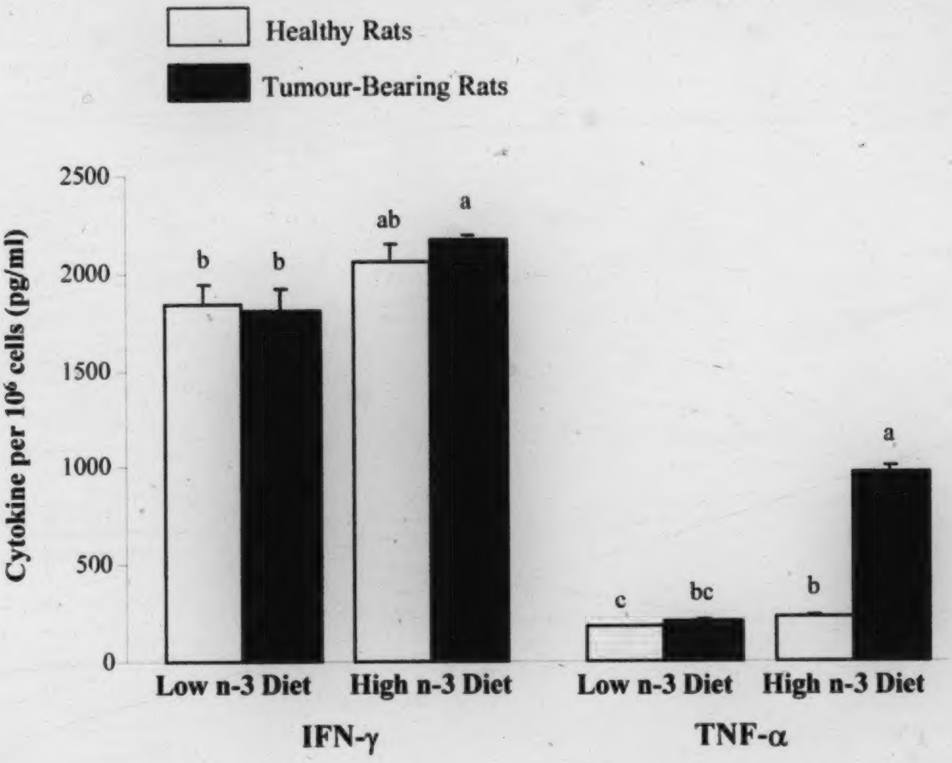

**FIGURE 6.4 Effect of Long-Chain n-3 Fatty Acids and Tumour Burden on Splenocyte Interferon-γ and Tumour Necrosis Factor-α Production.** Splenocytes were cultured with Phorbol Myrisate Acetate (PMA, 30 μg/L) plus Ionomycin (0.75 μmol/L) for 48 h. Concentrations of interferon-γ (IFN-γ) and tumour necrosis factor-α (TNF-α) in cell culture supernatants were determined by enzyme-linked immunosorbent assay as described in Materials and Methods. Bars represent means ± SEM ($n \geq 7$/group). The effects of diet and tumour were analyzed by two-way ANOVA. For each culture condition, bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

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# 7. MOLECULAR MECHANISMS OF THE EFFECT OF FATTY ACIDS ON MDA-MB-231 BREAST CANCER CELL GROWTH

## 7.1. INTRODUCTION

High levels of fish oil-derived long-chain n-3 fatty acids, eicosapentaenoic (C20:5n-3, EPA) and docosahexaenoic (C22:6n-3, DHA) acids, inhibit the growth of certain animal tumours compared with high levels of n-6 fatty acids, such as linoleic acid (C18:2n-6, LA) (Rose and Connolly 1999). The ability of long-chain n-3 fatty acids to inhibit tumour cell growth *in vitro* has also been well documented (Calviello et al. 1998, Awad et al. 1996, Begin et al. 1986). However, the cellular and molecular changes induced by n-3 fatty acids remain poorly understood. For example, it has not been established whether n-3 fatty acids exert their inhibitory effects by a reduction in tumour cell proliferation or an increase in tumour cell death (either through necrosis or apoptosis). Since the growth of a tumour depends on the balance between cell proliferation and cell loss (Lundberg and Weinberg 1999), it is possible that fatty acids affect both proliferation and death.

Apoptosis, or programmed cell death, is a biochemical process in which cells are programmed to die under a range of physiological and developmental factors (Saikumar et al. 1999). Many intracellular mediators have been identified which modulate apoptosis, such as cell cycle regulatory proteins, p53, the Bcl family of proteins, and Fas-associated death domain (Saikumar et al 1999). Apoptosis is believed to be one of the most important events governing the behaviour and fate of cells in the body and is of central importance to tumourigenesis. In cancer cells, the lack of an appropriate apoptotic signal or a defect in the mechanisms leading to induction of apoptosis results in deregulated growth control of cells (Lundberg and Weinberg 1999). Thus, methods aimed at promoting tumour cell apoptosis may have potential in cancer prevention and treatment.

Cell cycle control is the major regulatory mechanism of cell development and growth of all living organisms (Nurse 2000). The cell cycle can be subdivided into four phases: $G_1$, S, $G_2$, and M. S-phase (DNA synthesis phase) is the period during which the genome is replicated in preparation for division and M-phase (mitosis) is the period duing which the cell divides (Nurse 2000). The $G_1$ and $G_2$ phases follow upon mitosis and S-phase, respectively (Nurse 2000, Prescott 1976). Cells may also withdraw from the cell cycle into a quiesent state known as $G_0$ (Nurse 2000, Prescott 1976). In cancer, the cell cycle does not function properly, leading to uncontrolled cell proliferation, the basis for tumour formation (Sherr 1996). Thus, a characteristic feature of most, if not all, malignant tumours is deregulation of the cell cycle, which can occur on many levels, including loss of proper function of one or several of the proteins involved in cell cycle control (Landberg and Roos 1997, Sherr 1996). Clearly, preventing excessive cell proliferation through alterations in cell cycle progress or of the proteins involved in its control could have valuable implications for cancer treatment.

Among the principal components of the machinery regulating cell proliferation are the cyclin dependent kinases (cdks) and the cyclins (Nurse 2000, Lundberg and Weinberg 1999). The cdks are present throughout the cell cycle, but become active only when they interact with their cyclin partners (Nurse 2000, Lundberg and Weinberg 1999). Active cyclin:cdk complexes drive cells through particular cell cycle phases, by phosphorylating essential protein substrates (Nurse 2000, Lundberg and Weinberg 1999).

In general, cyclins may be divided into $G_1$ cyclins and mitotic cyclins, which are involved in the $G_1$-S and $G_2$-M transition, respectively (Dou et al. 1993, Koff et al. 1991). The D-type cyclins (D1, D2, D3) appear to promote $G_0$ to $G_1$ transitions and increase the rate of $G_1$ progression (Resnitzky et al. 1994). Cyclin B1, a mitotic cyclin synthesized during late S phase, is critical for progression through M phase (Darzynkiewicz et al. 1996). In noncancerous cells, cyclin proteins are synthesized and degraded in an orderly, scheduled manner within the cell (Nurse 2000, Lundberg and Weinberg 1999). However, in cancer cells this is not always the case. For example, breast cancer cells often produce excess of cyclin D (Pines 1995).

A wide array of other proteins are also involved in cell proliferation. For example, proliferating cell nuclear antigen (PCNA) is synthesized in early $G_1$ and S phases of the cell cycle and is essential for chromosomal DNA replication (Woods et al. 1991, Waseem and Lane 1990). Another protein, PRK (proliferation-related kinase) is a serine/threonine kinase whose expression is tightly regulated at various levels during different stages of the cell cycle and appears to play an important role in regulating the onset and/or progression of mitosis in mammalian cells (Ouyang et al. 1997). Other components of the cell cycle machinery are involved in arresting cell cycle progression. For example, the *retinoblastoma* gene encodes a nuclear phosphoprotein (Rb) which is present in normal cycling cells and acts as a tumour suppressor, serving as part of a braking mechanism acting on the cell cycle (Picksley and Lane 1994). If this braking mechanism becomes disabled, this can lead to unconstrained cell proliferation. Other molecules function in restraining cell cycle progression in extraordinary circumstances, as elements of checkpoint controls in response to physiological problems in the cell. For example, the *p53* gene encodes a 53 kDa nuclear phosphoprotein which normally functions in the context of DNA damage as a cell cycle checkpoint regulator to slow cell growth and induce DNA repair (Kastan et al. 1995). If damage is too severe for repair, cell death (apoptosis) is triggered (Kastan et al 1995).

A properly functioning cell cycle machinery is critical to normal cell growth and division. In recent years there has been an increased focus on connections between cell cycle control mechanisms and cancer development (Hanahan and Weinberg 2000, Landberg and Roos 1997, Pines 1995, Hartwell and Kastan 1994). Owing to the proposed abundance of cell cycle alterations in tumours, cell cycle defects might even be obligatory in cancer development {Strauss, Lukas, et al. 1995 ID: 1968}{Nielsen, Emdin, et al. 1997 ID: 1044}. Overall, the net effect of any deleterious changes in the cell cycle machinery is deregulation of the cell cycle and, in turn, excessive cell proliferation. Cyclins, cell cycle regulatory proteins, such as p53 and pRb, and proliferation-related proteins, such as PRK and PCNA, are logical targets for investigation into the mechanisms of the effect of fatty acids on tumour cell growth. As mentioned previously, inhibitory effects of n-3 fatty acids, including DHA, on human breast cancer cell lines have been reported (Calviello et al 1998, Awad et al 1996, Grammatikos et al. 1994, Begin et al 1986). We wanted to investigate potential cellular and molecular changes induced by n-3 fatty acids which may affect tumour growth. Since the antitumour effect of DHA may be due to a reduction in cell division, an increase in apoptosis, or both, we chose to investigate both mechanisms along with studying cell cycle distribution and the expression of various cell cycle components and growth-related proteins which may play a role in the proliferation and apoptosis processes. Furthermore, since alterations in membrane fatty acid composition can affect vital cell membrane functions which may be relevant to the relationship between fatty acids and tumour growth (Burns and Spector 1987), we also analyzed tumour cell membrane phospholipids important in intracellular signaling pathways.

## 7.2. MATERIALS AND METHODS

**Cell line maintenance.** The MDA-MB-231 human mammary adenocarcinoma cell line was obtained from the American Type Culture Collection (Rockville, MD) and maintained in 75 $cm^2$ tissue culture flasks (Corning Glass Works, Corning, NY) in Iscove's modified Dulbecco's medium supplemented with 1% (v/v) antibiotic/antimycotic solution (1 x $10^5$ U/L penicillin, 100 mg/L streptomycin, and 25 mg/L amphotericin B) and heat-inactivated fetal calf serum (FCS, 50 g/L) (all media components from Gibco BRL, Burlington, ON, Canada) at 37 °C in 5% $CO_2$ at 98% relative humidity. The cell culture media was changed every 3 d until monolayers reached 90% confluency (determined by viewing monolayers under an inverted microscope) at which time cells were passaged to new flasks containing fresh media.

**Preparation of fatty acid-supplemented media.** Docosahexaenoic acid (C22:6n-3, DHA) was purchased from Martek Biosciences Corporation (Columbia, MD). Linoleic acid (C18:2n-6, LA) was synthesized in our laboratory as previously described (Ma 1999). Preliminary experiments were performed to determine the fatty acid concentrations used in this study (Appendix C). The fatty acid concentrations chosen were 75 μM LA, 75 μM DHA, 75 μM LA + 75 μM DHA, and 150 μM LA, which were prepared from stock solutions of LA and DHA (1 mg/mL in ethanol) stored at –70 °C. The fatty acid-supplemented media was prepared by adding appropriate aliquots of fatty acid stock solution into 15 mL sterile tubes (Sarstedt Inc., Newton, NC). The ethanol in the aliquots was evaporated using $N_2(g)$ under sterile conditions. After the ethanol was evaporated, 1.25 mL of FCS was added to each tube containing fatty acid, vortexed, and incubated in a shaking water bath at 37 °C for 1 h. The tubes were vortexed every 15 min during the incubation. After 1 h, 10 mL of Iscove's modified Dulbecco's medium containing 1% (v/v) antibiotic/antimycotic solution was added to each tube, vortexed, and further incubated for 30 min in a shaking water bath at 37 °C. Following incubation, cell culture media was removed from each flask and replaced with the fresh media supplemented with fatty acid and FCS (11.25 mL). The remaining 14 mL of Iscove's modified Dulbecco's medium containing 1% (v/v) antibiotic/antimycotic solution was then added so that each flask contained 25.25 mL of media with the appropriate fatty acid concentration and 50 g/L FCS. Cells were incubated with fatty acid-supplemented media for 72 h, during which time the culture media was not changed.

**Cell count and viability.** Cells were seeded (1 x $10^6$ cells/flask) in culture media containing FCS (50 g/L) (described above) to allow for adherence and formation of monolayers. After 48 h, the standard cell culture media was replaced with fresh media containing fatty acids (described above) and cells were further incubated for 72 h. After 72 h, cells were harvested using trypsin-EDTA (Gibco BRL). Cell counts and viability were determined using a haemocytometer and the trypan blue (Sigma Chemical, St. Louis, MO) exclusion method. Cells were then washed in phosphate buffered saline (PBS, pH 7.4) containing FCS (10 g/L) and prepared for the assays described below or frozen as cell pellets at –70 °C for subsequent Western blot and lipid analyses.

**Cell proliferation.** DNA synthesis of MDA-MB-231 breast cancer cells was assessed by [methyl-$^3$H]-thymidine incorporation. Cells were seeded (2 x $10^4$ cell/mL/well) in 24-well sterile, flat-bottom tissue culture plates (Corning) and grown for 48 h in culture media containing FCS (50 g/L) (described above) to allow for adherence and monolayer formation. After 48 h, the standard cell culture media was replaced with fresh media containing fatty acids (described above) and cells were further incubated for 72 h. At 4 h prior to harvesting, cells were pulsed with [methyl-$^3$H]-thymidine (5 μCi; Amersham, Ontario, Canada). Media was then gently removed from each well and cells were incubated with 300 μL of trypsin-EDTA (Gibco BRL) for 3 min at 37 °C to disrupt cell adhesion to the plate. PBS (600 μL) was then added to each well and mixed thoroughly to evenly suspend cells. Aliquots (200 μL in quadruplicate) were transferred to non-sterile, non-tissue culture treated 96-well V-bottom plates (Costar®, Cambridge, MA). Cells were immediately harvested onto glass fiber filter mats (Skatron, Suffolk, UK) with a multiwell harvester (Skatron, Lier, Norway), transferred to scintillation vials containing Ecolite® scintillation cocktail (ICN, Montreal, QB, Canada) and counted (2 min/sample) on a betacounter (Beckman 5000, Beckman Instruments, Palo Alto, CA). Total dpm for each 200 μL aliquot were used to determine [methyl-$^3$H]-thymidine incorporation and to estimate the proliferative response.

**Cyclin and DNA analysis.** After 72 h, MDA-MB-231 breast cancer cells were harvested from flasks and cell number and viability were determined as described above. Cells were then washed in PBS containing FCS (10 g/L) by centrifuging at 400 g for 10 min and aspirating the supernatant. Cells were fixed by adding 5-10 mL of cold (–20 °C) 100% methanol, drop by drop, while vortexing vigorously to prevent cell clumping. Cells were then incubated at –20 °C for a minimum of overnight (and never longer than 30 d) prior to cyclin and DNA analysis. Following fixation (just prior to staining), methanol was removed from the samples by centrifugation at 400 g for 10 min. To ensure complete removal of methanol, tubes were turned upside down and patted on paper towel. Cells were then washed once in PBS containing FCS (10 g/L) as described above. After washing, 5 mL of cold 0.25% (v/v) Triton X-100 (BDH Chemicals, Toronto, ON, Canada) in PBS containing FCS (10 g/L) was added to the cell pellet, vortexed, and incubated for 5 min on ice. Cells were then washed once in PBS containing FCS (10 g/L) (described above) and the pellet was resuspended in 1 mL of PBS containing FCS (10 g/L). Cells were aliquoted into 12 x 75 mm round bottom tubes (Falcon®, Becton Dickinson, Franklin Lakes, NJ) for staining (approximately 1 x $10^6$ cells/tube). The cells were incubated in the dark for 30 min at room temperature with 20 μL of appropriately diluted mouse monoclonal antibody to fluorescein isothiocyanate (FITC)-conjugated mouse anti-human cyclins D1, D2, and B1 (PharMingen, San Diego, CA, USA; clones G124-326, G132-43, and GNS-1, respectively). The antibodies were diluted in PBS containing FCS (10 g/L) to obtain 0.5 μg of monoclonal antibody in a 20 μL volume. After incubation, cells were washed once in 2 mL of PBS containing FCS (10 g/L) by centrifugation for 5 min at 400 g. For simultaneous analysis of DNA synthesis and cyclin expression, the cell pellet was resuspended in 0.5 mL of propidium iodide solution (PI, 10 μg/mL in PBS; Sigma) and incubated at 4 °C in the dark for at least 10 min prior to analysis by flow cytometry. Cells were always analyzed within 2 hr of PI staining. If PI was not used, the cell pellet was resuspended in 0.5 mL of PBS. The control was prepared as described above, except that 20 µL of appropriately diluted FITC-conjugated mouse IgG1 monoclonal isotype-specific antibody (PharMingen, clone MOPC-21) was used instead of the anti-cyclin antibodies. Cellular fluorescence was measured using the FACScan flow cytometer (Becton Dickinson, Sunnyvale, CA, USA) and analyzed using Cell Quest software (Becton Dickinson).

**TdT-mediated dUTP Nick End Labeling (TUNEL).** Cells were washed three times in PBS, adjusted to 2 x $10^7$ cells/mL, and transferred into a V-bottom 96 well microtiter plate (100 µL/well, Costar®). Cells were fixed by adding 100 µL/well of a freshly prepared paraformaldehyde solution (40 g/L in PBS, pH 7.4), mixing well, and incubating on a shaker (to prevent extensive cell clumping) for 1 h at room temperature. After 1 h, fixative was removed by centrifugation at 300 g for 10 min and aspirating the supernatant. Cells were then washed once in PBS, resuspended in 100 µL/well permeabilisation solution (0.1% Triton X-100 in 0.1% sodium citrate), and incubated for 2 min on ice. Cells were then washed twice with PBS, resuspended in 50 µL/well TUNEL reaction mixture or in 50 µL/well TUNEL label (negative control), covered, and incubated for 60 min at 37 °C in a humidified atmosphere in the dark. For the positive control, fixed and permeabilized cells were first incubated with DNase 1 (1 mg/mL-1 µg/mL, 10 min at room temperature) to induce DNA strand breaks prior to adding the TUNEL reaction mixture. Following incubation, cells were washed three times in PBS and were diluted approximately 1:10 in PBS prior to acquisition and analysis by flow cytometry using Cell Quest software (Becton Dickinson).

**Cell lysates and protein determination.** Cells were harvested, collected in ice cold PBS, counted using a haemocytometer, and pelleted at 228 g. To prepare whole cell lysates, cells were resuspended in 250 µL of modified radioimmunoprecipitation assay (RIPA) buffer with freshly added protease inhibitors and incubated for 1 h on ice. Cell lysates were then centrifuged at 10 000 g for 10 min at 4 °C and the supernatant was stored at –20 °C. The protein concentration of cell lysates was determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

**SDS-PAGE.** MDA-MB-231 cancer cell lysates were separated via SDS-polyacrylamide gel electrophoresis (SDS-PAGE), according to the basic protocol of Laemmli (1970). Gels were cast and run using a Mini-PROTEAN® II Dual Slab Cell Apparatus (Bio-Rad Laboratories, Hercules, CA). Separating gels consisted of 0.07% (v/v) N, N, N', N'-tetramethyl-ethylenediamine (TEMED), 0.68% (v/v) ammonium persulfate (from a freshly made 10% w/v stock solution), 1.5 M Tris-HCl (pH 8.8), 1% v/v of 10% w/v SDS, and enough of a 30% acrylamide/1% bis-acrylamide solution to bring the level of acrylamide to the required percentage. Separating gels were layered with water and allowed to polymerize for a minimum of 1 h (or overnight) before pouring of the stacking gel, which contained 0.1% (v/v) TEMED, 1% (v/v) ammonium persulfate, 0.5 M Tris-HCl (pH 6.8), 1% (v/v) SDS, and the appropriate amount of a 30% acrylamide/1% bis-acrylamide solution. Stacking gels were allowed to polymerize for a minimum of 1 h. Gels were run in a buffer consisting of 25 mM Tris, 192 mM glycine, and 0.1% SDS. Rainbow™ (Amersham) coloured molecular weight standards were used to monitor protein separation. Samples (20 μg isolated protein) were diluted with 4x sample buffer (0.24 M Tris-HCl, 40% glycerol, 8% v/v of 10% w/v SDS, 0.5 mL bromophenol blue). Prior to gel loading, all samples and markers were heated at 95 °C for 5 min and centrifuged quickly to pellet. The gel was run at 80 mAmp until the desired marker distribution was achieved.

**Western blot analysis.** Proteins separated via SDS-PAGE were transferred to a nitrocellulose membrane (Nitropure, MSI Westboro, MA) using a Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad®). Transfer was carried out at 40 mAmp overnight in a buffer consisting of 25 mM Tris, 192 mM glycine, and 20% (v/v) methanol. Afte. transfer, the membrane was immersed in Ponceau S Solution (Sigma) with shaking for 5 min to confirm loading consistency and protein transfer. The membrane was then destained by rinsing with distilled $H_20$. The membrane was blocked for 2 h on a rotator at room temperature with Tris-Buffer Saline (TBS, pH 7.6) containing

0.1% (v/v) polyoxyethylene-sorbitan monolaurate (Tween-20, Sigma) and 5% (w/v) powdered milk (Lucerne Foods Ltd, Vancouver, BC, Canada). After blocking, the membrane was washed five times with 50 mL rinses of TBS containing 0.1% (v/v) Tween-20 (TBS-T): twice quickly, once for 15 min, and twice for 5 min. The membrane was then incubated on a rotator for 1 h at room temperature with the primary antibody diluted in TBS-T containing 1% (w/v) powdered milk. The membrane was washed as described above and then incubated with diluted secondary antibody in the same manner for 1 h. Following secondary antibody incubation, the membrane was washed again, with 2 additional 5 min washes, using fresh wash buffer each time. Secondary antibodies were detected using the Enhanced Chemiluminescence (ECL™) detection kit (Amersham) and membrane exposure to Kodak™ x-ray film (Eastman Kodak Company, Rochester, NY). Antibodies used in immunoblotting are described below. DO-7 is a monoclonal antibody (purified mouse anti-human) which recognizes an epitope between amino acids 1 and 45 of all known forms of human p53 (PharMingen, Mississauga, ON, Canada). PC10: *sc-56* is a mouse monoclonal antibody that reacts with the proliferating cell nuclear antigen (PCNA) p36 kDa protein expressed at high levels in proliferating cells (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). B37-2 is a monoclonal antibody (purified mouse anti-PRK) which reacts with human proliferation-related kinase (PharMingen). G3-245 is a monoclonal antibody (purified mouse anti-human) which recognizes an epitope between amino acids 300-380 of the human retinoblastoma protein (pp110-114 Rb) (PharMingen). All primary antibodies were used at 1 μg/mL. In all cases, the secondary antibody was an affinity-purified horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin specific polyclonal antibody used at a 1:10 000 dilution (PharMingen). The relative intensities of Western blot signals were determined using laser densitometry (Model GS-670 Imaging Densitometer, Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) and Molecular Analyst® Software (Version 2.1, Bio-Rad).

**Tumour cell fatty acid analysis.** Lipids were extracted from MDA-MB-231 breast cancer cells by a modified Folch (Folch et al. 1957) procedure (Field et al. 1988) using the same method described for splenocytes in Chapter 3. Individual phospholipids were separated on Whatman HPK thin layer chromatography plates (10 x 10 cm) and compared with appropriate standards as described in Chapter 3. Phosphatidylcholine (PC) and phosphatidylinositol (PI) fatty acid methyl esters were prepared using 14% (w/v) BF$_3$/methanol reagent (Morrison and Smith 1964) and separated by automated gas liquid chromatography as described in Chapter 3.

**Statistical analysis.** Results are presented as means ± SEM. All statistical analyses were conducted using the SAS statistical package (Version 6.11, SAS Institute, Cary, NC). Data were analyzed by one-way analysis of variance procedure (ANOVA) followed by a Duncan's multiple range test to identify significant ($p < 0.05$) differences between fatty acid treatments (Steele and Torrie 1980).

## 7.3. RESULTS

### MDA-MB-231 breast cancer cell growth

**(1) Cell counts.** MDA-MB-231 human breast cancer cells incubated for 72 h with 75 μM DHA had a significantly lower (-19%) number of cells per flask compared with cells cultured with 75 μM LA (**Figure 7.1**). In addition, incubation of cells with 75 μM DHA in the presence of 75 μM LA (LA + DHA) also resulted in a significant decrease (-25%) in the number of cells per flask compared with incubation with 75 μM LA alone (Figure 7.1). There was no significant difference in the number of cells per flask between cells cultured with DHA alone and cells cultured with DHA in the presence of LA (Figure 7.1). The growth inhibitory effects of DHA (with or without added LA in the media) do not appear to be due to a toxicity effect of the fatty acids, since cell viability (determined by trypan blue exclusion) was similar for all treatment groups. Finally, there was no significant difference in the number of cells per flask between cells cultured with 75 μM LA or 150 μM LA (Figure 7.1), suggesting that the suppressive effect of LA + DHA was not due to the total amount of fatty acid in the media (150 μM) since 150 μM LA did not have a growth inhibitory effect.

**(2) Cell proliferation.** MDA-MB-231 breast cancer cell growth was also determined by assessing [methyl-$^3$H]-thymidine incorporation which provides a measure of DNA synthesis. Using this method, the effect of DHA incubation on cell growth was greater than that determined by cell counting and trypan blue exclusion described above. [$^3$H]-thymidine incorporation by MDA-MB-231 breast cancer cells was significantly decreased (-38%) when cells were cultured for 72 h with 75 μM DHA compared with cells incubated with 75 μM LA (**Figure 7.2**). In addition, incubation of cells with 75 μM DHA in the presence of 75 μM LA (LA + DHA) also resulted in a significant decrease (-61%) in [$^3$H]-thymidine uptake by MDA-MB-231 cells (Figure 7.2). Furthermore, cell proliferation was significantly lower (-37%) when cells were incubated with LA + DHA compared with those cultured with DHA alone (Figure 7.2). This growth inhibitory effect does not appear to be due to the total amount of fatty acid in the media (150 μM) since there was no significant difference in [$^3$H]-thymidine uptake between cells cultured with 150 μM LA or 75 μM LA (Figure 7.2).

**DNA analysis.** The distribution of MDA-MB-231 breast cancer cells in the cell cycle phases ($G_0/G_1$, S or $G_2/M$) was not significantly different between fatty acid treatments (**Figure 7.3**). The proportion of tumour cells in $G_0/G_1$, S phase and $G_2/M$ was 75 ± 1%, 12 ± 1% and 14 ± 1%, respectively (*n* = 28/group).

**Expression of cyclin proteins.** MDA-MB-231 breast cancer cells expressed cyclins D1, D2 and B1 in all phases ($G_0/G_1$, S and $G_2/M$) of the cell cycle (**Table 7.1**). Expression of total (all cell cycle phases combined) cyclin D1, D2, and B1 within MDA-MB-231 breast cancer cells was not significantly different between fatty acid treatments. Overall, 27 ± 3% of MDA-MB-231 cells were cyclin D1 positive, 65 ± 3% were cyclin D2 positive and 26 ± 1% expressed cyclin B1. Expression of cyclins D1 and D2 within specific cell cycle phases was not significantly different between fatty acid treatments (Table 7.1). The proportion of cyclin B1 positive MDA-MB-231 tumour cells in S phase was significantly lower (-26%) in cells cultured with LA + DHA compared with those incubated with DHA alone (Table 7.1). Cyclin B1 expression in the other cell cycle phases was not significantly affected by fatty acid treatment (Table 7.1).

**Proliferation-related proteins**

**(1) PCNA.** The abundance of PCNA in MDA-MB-231 breast cancer cells did not differ between fatty acid treatment groups (**Figure 7.4**).

**(2) PRK.** The abundance of PRK in MDA-MB-231 breast cancer cells did not differ between fatty acid treatment groups (**Figure 7.5**).

**Cell cycle regulatory proteins**

**(1) p53.** The abundance of p53 was highest in MDA-MB-231 breast cancer cells incubated with LA + DHA compared with the other treatment groups (**Figure 7.6**). The greatest difference (+44% increase) in p53 expression was observed between cells incubated with 75 μM LA and those cultured with LA + DHA (Figure 7.6)

**(2) pRb.** The abundance of pRb in MDA-MB-231 breast cancer cells was significantly increased (+31%) when cells were cultured for 72 h with 75 μM DHA compared with cells incubated with 75 μM LA (**Figure 7.7**). In addition, incubation of cells with 75 μM DHA in the presence of 75 μM LA (LA + DHA) also resulted in a significant increase in the expression of pRb in MDA-MB-231 cells compared with cells incubated with 75 μM LA (+31%) or those cultured with 150 μM LA (+28%) (Figure 7.7). There was no significant difference the abundance of pRb between cells cultured with DHA alone and cells cultured with DHA in the presence of LA (Figure 7.7). Finally, there was no significant difference in pRb expression between cells cultured with 75 μM LA or 150 μM LA (Figure 7.7).

**Apoptosis.** MDA-MB-231 breast cancer cells cultured for 72 h with fatty acids contained a very small apoptotic subpopulation and the % of cells undergoing apoptosis cells did not differ between fatty acid treatment groups (0.24% ± 0.04, *n* = 15).

**MDA-MB-231 Breast Cancer Cell Phospholipid Analysis**

**(1) Phosphatidylcholine**

**(a) 75 μM LA vs 75 μM DHA.** MDA-MB-231 breast cancer cells incubated with DHA had a significantly higher % of saturated fatty acids (C14:0 and C16:0) and n-3 fatty acids [C20:5(3), C22:5(3) and C22:6(3)] in PC compared with cells incubated with LA (**Table 7.2**). Specifically, there was a 15-fold increase in the % of C22:6(3) and a 9-fold increase in total n-3 fatty acids in cells incubated with DHA compared with LA (Table 7.2). MDA-MB-231 cells incubated with DHA also had a significantly lower % of n-6 fatty acids [C18:2(6), C20:2(6), C20:3(6), C20:4(6), C22:4(6) and C22:5(6)], as well as significantly decreased monounsaturated fatty acids 16:1(7), C18:1(9), C18:1(7) and C24:1(9) in PC compared with cells incubated with LA (Table 7.2). In particular, the % of C18:2(6) was 6.5-fold higher in cells incubated with LA compared with those cultured with DHA (Table 7.2). The % of C18:0 and C20:3(9) in PC were also significantly lower in tumour cells cultured with DHA compared with LA (Table 7.2).

**(b) 75 μM LA vs LA + DHA.** MDA-MB-231 breast cancer cells cultured with LA + DHA had a significantly higher % of saturated fatty acids (C14:0 and C16:0) and n-3 fatty acids [C20:5(3), C22:5(3) and C22:6(3)] in PC compared with cells incubated with LA alone (Table 7.2). Specifically, there was an approximate 9-fold increase in the % of C22:6(3) and a 6-fold increase in total n-3 fatty acids in cells incubated with LA + DHA compared with those cultured with LA alone (Table 7.2). MDA-MB-231 cells incubated with LA + DHA also had a significantly lower % of n-6 fatty acids [C20:2(6), C20:3(6), C20:4(6), C22:4(6) and C22:5(6)], as well as significantly decreased monounsaturated fatty acids 16:1(7), C18:1(9) and C18:1(7) in PC compared with cells incubated with LA alone (Table 7.2). There was no significant difference in the % of C18:2(6) between cells incubated with LA alone or those cultured with LA + DHA (Table 7.2). The % of C18:0 and C20:3(9) in PC were also significantly lower in tumour cells cultured with LA + DHA compared with LA alone (Table 7.2).

**(c) 75 μM LA vs 150 μM LA.** MDA-MB-231 breast cancer cells incubated with 150 μM LA had an approximate 2-fold increase in the % of C18:2(6) and C20:2(6) in PC compared with cells incubated with 75 μM LA, although the % of C20:4(6) was significantly lower in cells cultured with the higher level of LA (Table 7.2). Overall, total n-6 fatty acids in PC increased approximately 1.5-fold when cells were incubated with 150 μM LA compared with 75 μM LA (Table 7.2). The n-6/n-3 and P/S ratios were also significantly increased when cells were incubated with the higher level of LA (Table 7.2). MDA-MB-231 cells incubated with 150 μM LA had a significantly lower % of monounsaturated fatty acids [C16:1(7), C16:1(5), C18:1(9) and C18:1(7)] in PC compared with cells incubated with 75 μM LA (Table 7.2) .

**(d) 75 μM DHA vs LA + DHA.** MDA-MB-231 breast cancer cells incubated with LA + DHA had a significantly higher % of n-6 fatty acids [C18:2(6) and C20:2(6)], C18:0 and P/S ratio in PC compared with cells incubated with DHA alone (Table 7.2). Specifically, the % of C18:2(6) was 6.5-fold higher in cells incubated with LA + DHA compared with those cultured with DHA alone (Table 7.2). MDA-MB-231 cells cultured with LA + DHA also had a significantly lower % of C20:4(6), n-3 fatty acids [C20:5(3) and C22:6(3)] and saturated fatty acids (C16:0) compared with cells incubated with DHA alone (Table 7.2) .

**(e) 150 μM LA vs LA + DHA.** MDA-MB-231 breast cancer cells incubated with LA + DHA had a significantly higher % of saturated fatty acids (C14:0 and C16:0) and n-3 fatty acids [C20:5(3), C22:5(3) and C22:6(3)] in PC compared with cells incubated with 150 μM LA alone (Table 7.2). In particular, total n-3 fatty acids increased approximately 7-fold when cells were incubated with LA + DHA compared to 150 μM LA alone (Table 7.2). MDA-MB-231 cells incubated with LA + DHA had a significantly lower % of monounsaturated fatty acids [C18:1(9) and C18:1(7)] and n-6 fatty acids [C18:2(6), C20:2(6), C20:3(6) and C22:4(6)] in PC compared with cells cultured with 150 μM LA alone (Table 7.2). Specifically, the % of C18:2(6) in PC was approximately 2-fold lower in cells incubated with LA + DHA compared with those cultured with 150 μM LA alone (Table 7.2). The % of C18:0 and C20:3(9) in PC were also significantly lower in tumour cells cultured with LA + DHA compared with 150 μM LA alone (Table 7.2).

**(2) Phosphatidylinositol**

**(a) 75 μM LA vs 75 μM DHA.** MDA-MB-231 breast cancer cells incubated with DHA had a significantly higher % of n-3 fatty acids [C20:5(3), C22:5(3) and C22:6(3)] in PI compared with cells incubated with LA (**Table 7.3**). Specifically, there was a 9-fold increase in the % of C22:6(3) and an approximate 6-fold increase in total n-3 fatty acids when cells were incubated with DHA compared with LA (Table 7.3). MDA-MB-231 cells cultured with DHA had a significantly lower % of C20:3(9), monounsaturated fatty acids [C18:1(9) and C18:1(7)] and n-6 fatty acids [C18:2(6), C20:3(6), C20:4(6), C22:4(6) and C22:5(6)] in PI compared with cells incubated with LA (Table 7.3). In particular, the % of C18:2(6) was approximately 3-fold higher in cells incubated with LA compared with those cultured with DHA (Table 7.3).

**(b) 75 μM LA vs LA + DHA.** MDA-MB-231 breast cancer cells cultured with LA + DHA had a significantly higher % of C18:2(6) and n-3 fatty acids [C20:5(3), C22:5(3) and C22:6(3)] in PI compared with cells incubated with LA alone (Table 7.3). Specifically, there was an approximate 6-fold increase in the % of C22:6(3) and an approximate 4-fold increase in total n-3 fatty acids in cells incubated with LA + DHA compared with LA alone (Table 7.3). MDA-MB-231 cells cultured with LA + DHA also had a significantly lower % of C20:3(9), monounsaturated fatty acids [C18:1(9) and C18:1(7)] and n-6 fatty acids [C20:3(6), C20:4(6), C22:4(6) and C22:5(6)] in PI compared with cells incubated with LA alone (Table 7.3).

**(c) 75 μM LA vs 150 μM LA.** MDA-MB-231 breast cancer cells incubated with 150 μM LA had an approximate 2-fold increase in the % of C18:2(6) and a 4-fold increase in the % of C20:2(6) in PI compared with cells incubated with 75 μM LA (Table 7.3). The total n-6 fatty acid content and n-6/n-3 ratio in PI was also significantly increased when cells were cultured with 150 μM LA compared with 75 μM LA, despite a significant decrease in the % of C22:4(6) and C22:5(6) when cells were incubated with the higher level of LA (Table 7.3). MDA-MB-231 cells incubated with 150 μM LA also had a significantly lower % of C18:0 and monounsaturated fatty acids [C18:1(9) and C18:1(7)] compared with cells cultured with 75 μM LA (Table 7.3).

**(d) 75 μM DHA vs LA + DHA.** MDA-MB-231 breast cancer cells incubated with LA + DHA had a significantly higher % of C18:0 and total n-6 fatty acids in PI compared with cells incubated with DHA alone (Table 7.3). In particular, the % of C18:2(6) was approximately 4.5-fold higher in cells cultured with LA + DHA compared with DHA alone (Table 7.3). MDA-MB-231 cells incubated with LA + DHA had a significantly lower % of n-3 fatty acids [C20:5(3), C22:5(3) and C22:6(3)] compared with cells incubated with DHA alone (Table 7.3).

**(e) 150 μM LA vs LA + DHA.** MDA-MB-231 breast cancer cells incubated with LA + DHA had a significantly higher % of C18:0 and n-3 fatty acids [C20:5(3), C22:5(3) and C22:6(3)] in PI compared with cells incubated with 150 μM LA alone (Table 7.3). In particular, total n-3 fatty acids in PI increased approximately 5-fold when cells were incubated with LA + DHA compared to 150 μM LA alone (Table 7.3). MDA-MB-231 cells cultured with LA + DHA had a significantly lower % of C20:3(9), monounsaturated fatty acids [C18:1(9) and C18:1(7)] and n-6 fatty acids [C18:2(6), C20:2(6), C20:3(6), C20:4(6) and C22:4(6)] in PI compared with cells incubated with 150 μM LA alone (Table 7.3). In particular, the % of C18:2(6) was approximately 1.5-fold lower in cells incubated with LA + DHA compared with those cultured with 150 μM LA (Table 7.3).

## 7.4. DISCUSSION

A number of studies using human breast cancer cells maintained in athymic nude mice or *in vitro* have demonstrated growth inhibitory effects of long-chain n-3 fatty acids, such as EPA and DHA (Chajes et al. 1995, Grammatikos et al 1994, Rose and Connolly 1990). We wanted to examine potential molecular mechanisms associated with the influence of DHA on tumour growth. Specifically, we determined if the antitumour effect of DHA was related to an inhibition in cell proliferation, an increase in apoptosis, and/or alterations in the cell cycle, including changes in expression of key cell cycle and growth-related proteins. Our model was the MDA-MB-231 cell line, a highly aggressive, estrogen receptor-negative human mammary adenocarcinoma cell line (Crepin et al.

1990). Although it is known that this cell line is responsive to various fatty acids supplemented in the media (Noguchi et al. 1995, Rose and Connolly 1990), it was still necessary to establish appropriate media conditions as cell growth may be affected by numerous factors, including fetal calf serum concentration (Mæhle et al. 1995), bovine serum albumin (desBordes and Lea 1995) and ethanol in the media (Angeletti and de Alaniz 1996). Since we were interested in examining tumour cell membrane phospholipids, and since ethanol is known to affect the cell membrane (Chen et al. 1996), we established media conditions in the absence of this compound. The concentrations of LA (75 μM) and DHA (75 μM) we used were based on preliminary work examining growth effects over a wide range of concentrations (2 μM – 300 μM). Previous studies have shown that MDA-MB-231 cell growth is stimulated by LA and inhibited by DHA and EPA at concentrations of approximately 3 μM (0.75 μg/mL) (Rose and Connolly 1990). Another study found that growth of this cell line was inhibited by 0.1-8.1 μg/mL DHA (<1-25 μM) in the presence or absence of 2.2 μM LA (Noguchi et al 1995). A DHA concentration of 60 μM has also been reported to inhibit MDA-MB-231 breast cancer cell proliferation without inducing cytotoxic effects (Chajes et al 1995). Overall, cell culture conditions (media, level of bovine serum albumin and fetal calf serum, presence of ethanol) and the method of delivery of fatty acids we used differed in some aspects from those previously reported, which may have accounted for discrepancies in the levels of fatty acids used.

**Proliferation**

In the present study, LA (75 μM) resulted in the highest estimated rate of MDA-MB-231 cell proliferation, while incubation of breast cancer cells with 75 μM DHA, either alone or in the presence of 75 μM LA, inhibited tumour cell proliferation (Figure 7.2). In part, DHA-induced inhibition of thymidine uptake can be attributed to lower cell numbers (Figure 7.1) in the DHA-treated groups at the time the [methyl-$^3$H]-thymidine was added. However, this alone can not fully account for the inhibitory effect of DHA on MDA-MB-231 growth. Incubation of cells with DHA in the presence of LA resulted in the greatest decrease in tumour cell growth (Figure 7.2). Ip et al. (1985) have determined a LA requirement for tumour cell development and growth (Ip et al. 1985). In previous studies (e.g. Rose and Connolly 1990, Begin et al. 1988) it has been difficult to exclude the possibility that DHA-induced growth inhibition may be due to the absence of the essential growth factor, LA. For this reason, LA was included in one of the DHA treatment groups (LA + DHA) to ensure that inhibition of tumour cell proliferation by DHA was not merely due to the absence of LA in the cell culture media. However, since higher fatty acid concentrations added to the media may be toxic to cells (Rose and Connolly 1990, Begin et al. 1988), we had to ensure that supplementation of 75 μM LA + 75 μM DHA did not inhibit cell proliferation due to a fatty acid-induced cytotoxic effect. Thus, an additional control was used (150 μM LA) which contained an equal total fatty acid concentration to the LA plus DHA treatment group. The proliferation rate was not significantly different when the LA concentration was increased from 75 μM to 150 μM, suggesting that the addition of this level of fatty acids to the cell culture media did not induce a cytotoxic effect on the breast cancer cells. Furthermore, trypan blue exclusion indicated that the cells present were viable, suggesting that cell loss was not due to cytotoxic cell death. Chajes et al. (1995) also found that growth arrest of MDA-MB-231 breast cancer cells by EPA and DHA was not due to major cytotoxic effects.

The incorporation of dietary fatty acids into tumour cell membranes is well established (Calviello et al 1998, Noguchi et al. 1997, Rose et al. 1995, Karmali et al. 1984). In addition, it is known that the phospholipid composition of cells may be modified *in vitro* when different fatty acids are added to the cell culture media (Spector and Yorek 1985). As expected, MDA-MB-231 cells incubated with DHA had a higher total n-3 content and a lower n-6/n-3 ratio in PC and PI. It is not known whether DHA-induced changes in tumour cell lipid composition are directly associated with changes in second messenger pathways leading to growth inhibition of MDA-MB-231 tumour cells or if other cell functions, such as eicosanoid production are involved in our model. Dietary fat modulation of intracellular second messengers (Chapkin et al. 1997) and eicosanoids (Karmali 1987) have been reported in other studies. It is possible that changes in membrane lipid composition may modulate downstream events involved in cell proliferation, however this requires further study.

Our study shows that DHA does not appear to function simply by substituting for the growth-enhancing LA in the membrane. In fact, when LA was included in the media with DHA, cell proliferation was further reduced compared with cells cultured with DHA alone. These findings should be considered in association with specific changes in tumour cell membrane composition (Tables 7.2-7.3). MDA-MB-231 cells incubated with DHA had lower membrane levels of C18:2(6) in PC and PI compared with cells cultured with LA. This may have been associated with decreased proliferation in cells cultured with DHA, since LA has been shown to exist at higher concentrations in membranes of dividing cells (Burns and Spector 1994). However, cells cultured with LA + DHA had similar (or higher) membrane levels of C18:2(6) (compared with LA alone) and yet proliferation was markedly lower in this treatment group, suggesting that DHA did not merely substitute for LA in the membrane. Tumour cells incubated with DHA (either alone or with LA in the media) had lower C20:4(6) in PC and PI compared with cells cultured with LA alone, suggesting levels of this n-6 fatty acid may be associated with decreased proliferation. Furthermore, cells incubated with LA + DHA had lower C20:4(6) in PC compared with cells incubated with DHA alone which corresponded with reduced proliferation. MDA-MB-231 breast cancer cells express constitutive cyclooxygenase-2 and produce C20:4(6)-derived eicosanoids, such as prostaglandin E2 (Liu and Rose 1996). Recently, Connolly et al. (1999) reported that reduced tumour production of eicosanoids derived from cyclooxygenase-2 (prostaglandin E2) and lipoxygenase activity (reduced 12-hydroxyeicosatetraenoic acid) combined to suppress MDA-MB-231 tumour growth in DHA-fed animals. Interestingly, in our study, cells cultured with the higher level of LA (150 μM) had similar (or higher) membrane C20:4(6) compared with cells incubated with LA + DHA and yet there was no difference in proliferation between these two groups.

### Proliferation-Related Protein Expression

Since DHA inhibited tumour cell proliferation, we hypothesized that DHA (either in the absence or presence of LA) may have altered expression of certain growth-related proteins within tumour cells. A wide array of intracellular proteins are involved in cell proliferation (Shackney and Shankey 1999), including PCNA and PRK. PCNA is a 36 kDa molecular weight protein synthesized in early $G_1$ and S phases of the cell cycle and is essential for chromosomal DNA replication (Woods et al 1991, Waseem and Lane

1990). PRK (proliferation-related kinase) is a serine/threonine kinase that appears to play an important role in regulating the onset and/or progression of mitosis in mammalian cells and its expression is tightly regulated at various levels during different stages of the cell cycle (Ouyang et al 1997, Li et al. 1996). In our study, MDA-MB-231 tumour cells expressed both of these growth-related proteins. However, we found that the fatty acids we incubated did not significantly affect expression of either PCNA or PRK in MDA-MB-231 breast cancer cells (Figures 7.4-7.5), suggesting that these proteins are not involved in DHA-induced inhibition of this cell line.

### Cell Cycle Phase Distribution and Cyclin Protein Expression

Cell cycle control is the key regulatory mechanism of cell growth (Nurse 2000). Thus, we hypothesized that the observed changes in growth rate when MDA-MB-231 cells were incubated with DHA (or LA + DHA) may reflect changes in cell cycle control. This led us to examine possible effects of LA and DHA on the distribution of cells within the cell cycle phases and expression of cell cycle-related proteins, such as cyclins, within the tumour cells. It was previously shown that EPA-induced growth inhibition of a pancreatic cancer cell line (M1A PaCa-2) *in vitro* may have been mediated in part via cell cycle arrest (Lai et al. 1996). We hypothesized that the inhibitory effect of DHA (and LA + DHA) on tumour cell proliferation was associated with cell cycle arrest and an accumulation of tumour cells in a particular phase of the cell cycle: e.g $G_0$ (quiescent phase). However, neither DHA nor LA + DHA altered the distribution of tumour cells in the cell cycle phases ($G_0/G_1$, S or $G_2/M$) compared with cells cultured with LA alone (Figure 7.3). Cyclins are key components of the cell cycle progression machinery (Nurse 2000, Lundberg and Weinberg 1999). A variety of cyclin-cdk complexes are formed during distinct phases of the cell cycle, each dedicated to the phosphorylation of specific sets of target proteins (Lundberg and Weinberg 1999). During unperturbed growth of normal cells, cyclin proteins are synthesized and degraded in an orderly manner, at discrete and well-defined periods of the cell cycle (Darzynkiewicz et al 1996). Immunocytochemical detection of cyclins in relation to cell cycle position (DNA content) by multiparameter flow cytometry can be used to detect unscheduled appearance of cyclins, namely, the expression of $G_1$ cyclins by cells in $G_2/M$ and of $G_2/M$ cyclins by $G_1$

cells, without the need for cell synchronization (Darzynkiewicz et al 1996). Since cyclins D1 and D2 are predominately found in $G_0/G_1$ phase of the cycle, noncancerous cells would express little, if any, D-type cyclins in S and $G_2/M$ phases. MDA-MB-231 tumour cells expressed some cyclin D1 and D2 in $G_2/M$ and S phases of the cycle which was not surprising given that unscheduled expression of cyclins in certain tumour cell lines is associated with disregulation of cell cycle machinery (Hanahan and Weinberg 2000). However, there were no significant effects of the fatty acids we incubated on cyclin D1 or D2 expresssion in specific cell cycle phases (Table 7.1). As expected, cyclin B1 expression in MDA-MB-231 cells was highest during S and $G_2/M$ phases (Darzynkiewicz et al 1996). Tumour cells incubated with LA + DHA had significantly lower (-26%) expression of cyclin B1 in S phase compared with cells cultured with DHA alone (Table 7.1). Thus, MDA-MB-231 cells cultured with LA + DHA had the lowest rate of proliferation as well as reduced expression of a mitotic cyclin, which is necessary for progression of cells through mitosis.

**Cell Cycle Regulatory Protein Expression**

The *p53* gene, which encodes a 53 kDa nuclear phosphoprotein, is the most commonly mutated gene yet identified in human cancers (Vogelstein 1990), with half of all types of tumours lacking a functional p53 protein (Kirsch and Kastan 1998). Our results showed that expresssion of p53 in MDA-MB-231 cancer cells was 44% higher after cells were incubated with DHA (in the presence of LA) (Figure 7.6) At a molecular level, p53 acts as a tumour suppressor to inhibit cell proliferation when DNA damage occurs (Picksley and Lane 1994). Specifically, p53 induces growth arrest in multiple points of the cell cycle through induction of a cdk inhibitor, p21, which ultimately arrests the cell cycle (Harper et al. 1993, el-Deiry et al. 1993). If damage is too severe for repair, cell death (apoptosis) is triggered (Kastan et al 1995). Thus, p53 acts as a master governor of two distinct downstream machineries: it may inhibit cell cycle progression or may induce apoptosis. It is possible that the growth inhibitory effect of DHA may be associated with increased p53 tumour suppressor protein expression. However, since we did not determine the functional activity of p53 expressed in MDA-MB-231 cells, we cannot currently explain the significance and mechanism of increased p53 following LA + DHA supplementation.

To our surprise, we found that MDA-MB-231 breast cancer cells cultured for 72 h with DHA contained a very small apoptotic subpopulation (< 1%) and there were no significant differences between cells cultured with LA. There is evidence that long-chain n-3 fatty acids can induce apoptosis (Connolly et al. 1999, Calviello et al 1998, Lai et al 1996, Gabor and Abraham 1986). A study by Gabor & Abraham (1986), which predated the current intense interest in apoptosis and cancer, found that growth inhibition of a transplantable mouse mammary adenocarcinoma by dietary n-3 fatty acids resulted from cell loss, rather than suppressed proliferation. Recently, Connolly et al. (1999) showed that DHA-induced inhibition of MDA-MB-231 breast cancer cell growth *in vivo* was associated with both a partial suppression of cell proliferation and increased apoptosis, suggesting that the potential mechanism for DHA-induced inhibition is multifactorial. Again, although DHA upregulated p53 (an inducer of apoptosis) expression in our model, the functional significance of this effect needs to be determined.

Our results showed that expression of pRb, another tumour suppressor protein, was also increased after MDA-MB-231 tumour cells were incubated with DHA (either in the absence or presence of LA) (Figure 7.7). The phosphorylation state of pRb impacts upon the capacity of cells to proceed into S phase (Hollingsworth et al. 1993). The signal transduction events leading up to pRB phosphorylation and/or functional inactivation are disrupted in many and perhaps all types of cancer cells (Sherr 1993). In $G_1$, D-type cyclins bind to cdks 4 or 6 and the resulting complexes hyperphosphorylate pRb, releasing the braking effect of pRb and enabling the cell to progress into late $G_1$ and then into S phase (Sherr 1993). An underphosphorylated form of Rb is mainly found in resting or fully differentiated cells, whereas the hyperphosphorylated form is present in proliferating cells. Under conditions which threaten genomic integrity, p53 accumulates in a transcriptionally active form and induces expression of p21, which blocks the hyperphosphorylation of Rb and in turn inhibits activity of E2F transcription factors (Hatakeyama and Weinberg 1995). As with p53, our results cannot provide information about the functional activity of pRb. However, increases in both pRb and p53 expression in MDA-MB-231 cells incubated with DHA (with LA) suggest involvment of the tumour suppressor machinery in the observed growth inhibitory effects. To further understand the molecular mechanisms underlying DHA-induced reduction in breast cancer cell growth, future studies should focus more precisely on functional changes in these tumour suppressor proteins following fatty acid incubation, as well as the mechanism by which DHA upregulated p53 and pRb levels.

## Conclusions

In this study, DHA (either in the absence or presence of LA in the media) reduced MDA-MB-231 breast cancer cell proliferation but did not affect tumour cell apoptosis. Thus, inhibition of proliferation, rather than enhanced apoptosis, appears to be the mechanism by which DHA exerts its antitumour effect in our model. The growth inhibitory effect of DHA may be associated with DHA-induced modulation of the fatty acid composition of major membrane phospholipids, PC and PI, involved in intracellular signaling events, however further studies are required. Although DHA did not alter the distribution of tumour cells within the cell cycle phases or expression of D-type cyclins and proliferation-related proteins, levels of the mitotic cyclin B1 were decreased while tumour suppressor proteins, p53 and pRb were upregulated following DHA (with LA) incubation. Further work is needed to establish a relationship between DHA-induced changes in tumour cell membrane composition and downstream suppression of the mitotic cyclin B1 and upregulation of tumour suppressor proteins.

**TABLE 7.1 Expression of Cyclin Proteins in MDA-MB-231 Breast Cancer Cells After 72 h Incubation With Fatty Acids[1]**

| | 75 μM LA | 75 μM DHA | 75 μM LA + DHA | 150 μM LA |
|---|---|---|---|---|
| **Cyclin D1** | | | | |
| $G_0/G_1$ phase | 9.1 ± 1.7 | 8.9 ± 2.2 | 13 ± 4 | 8.6 ± 1.6 |
| S phase | 9.1 ± 2.5 | 6.9 ± 1.1 | 5.8 ± 1.2 | 5.0 ± 0.9 |
| $G_2$/M phase | 9.9 ± 0.7 | 9.8 ± 1.3 | 10 ± 2 | 9.2 ± 1.5 |
| **Cyclin D2** | | | | |
| $G_0/G_1$ phase | 48 ± 2 | 42 ± 5 | 46 ± 4 | 47 ± 3 |
| S phase | 10 ± 1 | 10 ± 1 | 8.8 ± 0.9 | 10 ± 1 |
| $G_2$/M phase | 12 ± 1 | 13 ± 1 | 12 ± 1 | 12 ± 1 |
| **Cyclin B1** | | | | |
| $G_0/G_1$ phase | 5.9 ± 1.0 | 5.5 ± 1.1 | 6.3 ± 1.8 | 2.7 ± 0.6 |
| S phase | 8.3 ± 0.9 | 9.3 ± 0.6 | 6.9 ± 0.9* | 7.0 ± 1.0 |
| $G_2$/M phase | 12 ± 1 | 13 ± 1 | 13 ± 2 | 12 ± 1 |

[1] Values are means ± SEM ($n \geq 4$ per treatment). LA, linoleic acid; DHA, docosahexaenoic acid

* indicates significantly different from 75 μM DHA treatment group ($p < 0.05$)

**TABLE 7.2 Fatty Acid Composition of Phosphatidylcholine of MDA-MB-231 Breast Cancer Cells Following Incubation with Various Fatty Acids[1]**

| | 75 μM LA | 75 μM DHA | 75 μM LA + DHA | 150 μM LA |
|---|---|---|---|---|
| **Fatty Acid** | % w/w of total fatty acids | | | |
| C14:0 | $2.0 \pm 0.1^{b}$ | $2.6 \pm 0.1^{a}$ | $2.7 \pm 0.1^{a}$ | $1.9 \pm 0.1^{b}$ |
| C16:0 | $25 \pm 0.4^{c}$ | $39 \pm 0.3^{a}$ | $34 \pm 0.7^{b}$ | $26 \pm 0.2^{c}$ |
| C16:1 (7) | $2.0 \pm 0.1^{a}$ | $1.2 \pm 0.03^{b}$ | $1.2 \pm 0.02^{b}$ | $1.3 \pm 0.5^{b}$ |
| C16:1 (5) | $1.2 \pm 0.1^{a}$ | $1.1 \pm 0.02^{a}$ | $1.1 \pm 0.02^{a}$ | $0.79 \pm 0.04^{b}$ |
| C17:0 | $0.78 \pm 0.01$ | $0.77 \pm 0.03$ | $0.81 \pm 0.03$ | $0.82 \pm 0.02$ |
| C18:0 | $19 \pm 0.4^{a}$ | $14 \pm 0.5^{c}$ | $16 \pm 0.2^{b}$ | $17 \pm 0.6^{a}$ |
| C18:1 (9) | $20 \pm 0.7^{a}$ | $10 \pm 0.2^{c}$ | $9.9 \pm 0.2^{c}$ | $12 \pm 0.3^{b}$ |
| C18:1 (7) | $4.1 \pm 0.04^{a}$ | $3.0 \pm 0.05^{c}$ | $2.9 \pm 0.03^{c}$ | $3.4 \pm 0.06^{b}$ |
| C18:2 (6) | $13 \pm 0.5^{b}$ | $2.0 \pm 0.04^{c}$ | $13 \pm 0.2^{b}$ | $24 \pm 0.8^{a}$ |
| C20:1 | $0.09 \pm 0.04$ | $0.08 \pm 0.03$ | $0.14 \pm 0.05$ | $0.07 \pm 0.03$ |
| C20:2 (6) | $1.9 \pm 0.16^{b}$ | $0.12 \pm 0.04^{d}$ | $0.84 \pm 0.03^{c}$ | $3.7 \pm 0.3^{a}$ |
| C20:3 (9) | $0.28 \pm 0.02^{a}$ | $0.03 \pm 0.02^{b}$ | nd $^{b}$ | $0.31 \pm 0.02^{a}$ |
| C20:3 (6) | $1.8 \pm 0.03^{a}$ | $0.81 \pm 0.02^{b}$ | $0.83 \pm 0.08^{b}$ | $1.6 \pm 0.05^{a}$ |
| C20:4 (6) | $3.0 \pm 0.1^{a}$ | $2.6 \pm 0.02^{b}$ | $2.1 \pm 0.1^{c}$ | $2.2 \pm 0.04^{c}$ |
| C20:5 (3) | $0.22 \pm 0.02^{c}$ | $2.9 \pm 0.2^{a}$ | $1.6 \pm 0.1^{b}$ | $0.30 \pm 0.06^{c}$ |
| C22:4 (6) | $0.68 \pm 0.08^{a}$ | $0.39 \pm 0.03^{b}$ | $0.33 \pm 0.02^{b}$ | $0.68 \pm 0.06^{a}$ |
| C22:5 (6) | $0.10 \pm 0.04^{a}$ | nd$^{c}$ | $0.02 \pm 0.01^{bc}$ | $0.08 \pm 0.02^{ab}$ |
| C22:5 (3) | $0.85 \pm 0.02^{b}$ | $2.5 \pm 0.1^{a}$ | $2.0 \pm 0.4^{a}$ | $0.65 \pm 0.04^{b}$ |
| C22:6 (3) | $1.1 \pm 0.04^{c}$ | $15 \pm 0.4^{a}$ | $9.7 \pm 0.4^{b}$ | $0.89 \pm 0.03^{c}$ |
| C24:1 (9) | $0.10 \pm 0.04$ | $0.03 \pm 0.02$ | $0.03 \pm 0.01$ | $0.13 \pm 0.04$ |
| SFA | $48 \pm 0.4^{c}$ | $57 \pm 0.3^{a}$ | $53 \pm 1^{b}$ | $47 \pm 1^{c}$ |
| MUFA | $27 \pm 1^{a}$ | $16 \pm 0.2^{c}$ | $15 \pm 0.2^{c}$ | $18 \pm 0.4^{b}$ |
| P/S ratio | $0.49 \pm 0.02^{c}$ | $0.46 \pm 0.01^{c}$ | $0.57 \pm 0.02^{b}$ | $0.73 \pm 0.04^{a}$ |
| n-6 PUFA | $21 \pm 1^{b}$ | $5.9 \pm 0.03^{d}$ | $17 \pm 0.2^{c}$ | $32 \pm 1^{a}$ |
| n-3 PUFA | $2.2 \pm 0.1^{c}$ | $20 \pm 0.4^{a}$ | $13 \pm 1^{b}$ | $1.9 \pm 0.1^{c}$ |
| n-6/n-3 | $9.5 \pm 0.3^{b}$ | $0.29 \pm 0.01^{c}$ | $1.3 \pm 0.1^{c}$ | $17 \pm 1^{a}$ |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 5$ per treatment). Abbreviations used: LA, linoleic acid; DHA, docosahexaenoic acid; nd, not detectable; SFA, sum of saturated fatty acids; MUFA, sum of monounsaturated fatty acids; n-6 PUFA, sum of n-6 polyunsaturated fatty acids; n-3 PUFA, sum of n-3 polyunsaturated fatty acids; P/S ratio, ratio of polyunsaturated to saturated fatty acids. Values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

**TABLE 7.3 Fatty Acid Composition of Phosphatidylinositol of MDA-MB-231 Breast Cancer Cells Following Incubation with Various Fatty Acids**[1]

| | 75 μM LA | 75 μM DHA | 75 μM LA + DHA | 150 μM LA |
|---|---|---|---|---|
| **Fatty Acid** | | % w/w of total fatty acids | | |
| C14:0 | 0.39 ± 0.10 | 0.52 ± 0.06 | 0.47 ± 0.04 | 0.62 ± 0.11 |
| C16:0 | 3.9 ± 0.3 | 4.2 ± 0.5 | 3.5 ± 0.2 | 4.0 ± 0.4 |
| C16:1 (7) | 0.25 ± 0.02 | 0.24 ± 0.02 | 0.27 ± 0.04 | 0.21 ± 0.01 |
| C16:1 (5) | 0.15 ± 0.01 | 0.16 ± 0.01 | 0.14 ± 0.01 | 0.12 ± 0.01 |
| C17:0 | 0.50 ± 0.01$^{b}$ | 0.57 ± 0.04$^{a}$ | 0.53 ± 0.02$^{ab}$ | 0.47 ± 0.01$^{b}$ |
| C18:0 | 47 ± 0.5$^{ab}$ | 46 ± 0.2$^{bc}$ | 47 ± 0.5$^{a}$ | 45 ± 0.3$^{c}$ |
| C18:1 (9) | 8.4 ± 0.4$^{a}$ | 4.6 ± 0.3$^{bc}$ | 4.4 ± 0.2$^{bc}$ | 5.3 ± 0.2$^{b}$ |
| C18:1 (7) | 2.1 ± 0.1$^{a}$ | 1.4 ± 0.05$^{c}$ | 1.3 ± 0.04$^{c}$ | 1.6 ± 0.03$^{b}$ |
| C18:2 (6) | 4.4 ± 0.2$^{c}$ | 1.3 ± 0.1$^{d}$ | 6.0 ± 0.2$^{b}$ | 9.8 ± 0.5$^{a}$ |
| C20:1 | 0.11 ± 0.03$^{b}$ | 0.29 ± 0.05$^{ab}$ | 0.33 ± 0.05$^{a}$ | 0.48 ± 0.12$^{a}$ |
| C20:2 (6) | 0.58 ± 0.11$^{b}$ | 0.67 ± 0.04$^{b}$ | 0.28 ± 0.07$^{b}$ | 2.4 ± 0.3$^{a}$ |
| C20:3 (9) | 0.92 ± 0.04$^{a}$ | 0.07 ± 0.02$^{b}$ | 0.18 ± 0.05$^{b}$ | 0.99 ± 0.04$^{a}$ |
| C20:3 (6) | 6.3 ± 0.7$^{a}$ | 2.6 ± 0.1$^{c}$ | 3.2 ± 0.1$^{bc}$ | 6.8 ± 0.3$^{a}$ |
| C20:4 (6) | 15 ± 1$^{a}$ | 12 ± 0.4$^{b}$ | 12 ± 0.4$^{b}$ | 14 ± 0.5$^{a}$ |
| C20:5 (3) | 0.42 ± 0.02$^{c}$ | 3.3 ± 0.3$^{a}$ | 1.9 ± 0.2$^{b}$ | 0.28 ± 0.05$^{c}$ |
| C22:4 (6) | 3.1 ± 0.2$^{a}$ | 1.1 ± 0.1$^{c}$ | 1.3 ± 0.1$^{c}$ | 2.4 ± 0.1$^{b}$ |
| C22:5 (6) | 0.53 ± 0.03$^{a}$ | 0.15 ± 0.03$^{c}$ | 0.17 ± 0.01$^{bc}$ | 0.25 ± 0.04$^{b}$ |
| C22:5 (3) | 1.8 ± 0.1$^{c}$ | 3.3 ± 0.2$^{a}$ | 2.5 ± 0.1$^{b}$ | 1.6 ± 0.1$^{c}$ |
| C22:6 (3) | 1.9 ± 0.2$^{c}$ | 17 ± 1$^{a}$ | 12 ± 0.5$^{b}$ | 1.7 ± 0.2$^{c}$ |
| C24:1 (9) | 0.15 ± 0.06 | 0.05 ± 0.02 | 0.06 ± 0.01 | 0.10 ± 0.02 |
| SFA | 53 ± 1 | 52 ± 1 | 53 ± 1 | 52 ± 1 |
| MUFA | 11 ± 0.5$^{a}$ | 6.7 ± 0.4$^{c}$ | 6.5 ± 0.3$^{c}$ | 7.8 ± 0.3$^{b}$ |
| P/S ratio | 0.67 ± 0.02$^{b}$ | 0.79 ± 0.03$^{a}$ | 0.76 ± 0.02$^{a}$ | 0.78 ± 0.02$^{a}$ |
| n-6 PUFA | 31 ± 1$^{b}$ | 18 ± 0.4$^{d}$ | 23 ± 0.5$^{c}$ | 36 ± 1$^{a}$ |
| n-3 PUFA | 4.1 ± 0.2$^{c}$ | 24 ± 1$^{a}$ | 17 ± 1$^{b}$ | 3.6 ± 0.3$^{c}$ |
| n-6/n-3 | 7.6 ± 0.5$^{b}$ | 0.75 ± 0.02$^{c}$ | 1.4 ± 0.1$^{c}$ | 10 ± 1$^{a}$ |

[1] Values are percentages of total fatty acids and are expressed as means ± SEM ($n \geq 5$ per treatment). Abbreviations used are defined in the legend to Table 7.2. Values within a row without a common superscript are significantly different ($p \leq 0.05$) as identified by a Duncan's multiple range test.

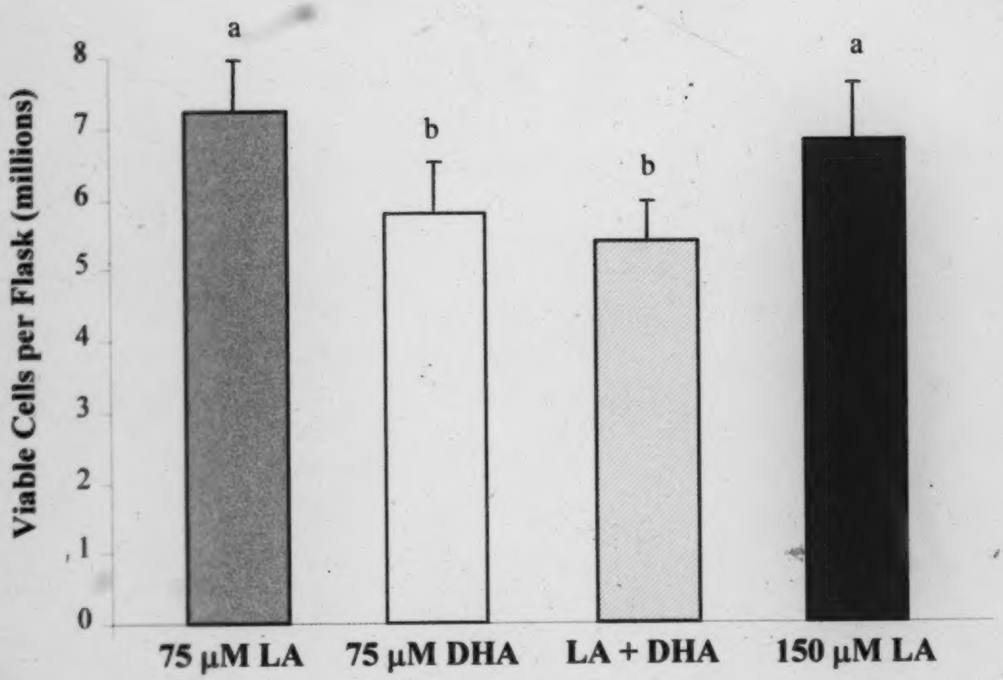

**FIGURE 7.1 Number of Viable MDA-MB-231 Breast Cancer Cells per Flask After 72 h Incubation With Fatty Acids.** Cells were seeded (1 x $10^6$ cells/flask) in culture media containing fetal calf serum (50 g/L) and allowed to adhere. After 48 h, the standard cell culture media was replaced with fresh media containing fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid). After 72 h, cells were harvested and cell counts and viability were determined using a haemocytometer and the trypan blue exclusion method. Bars represent means ± SEM (*n* = 5/treatment). Bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

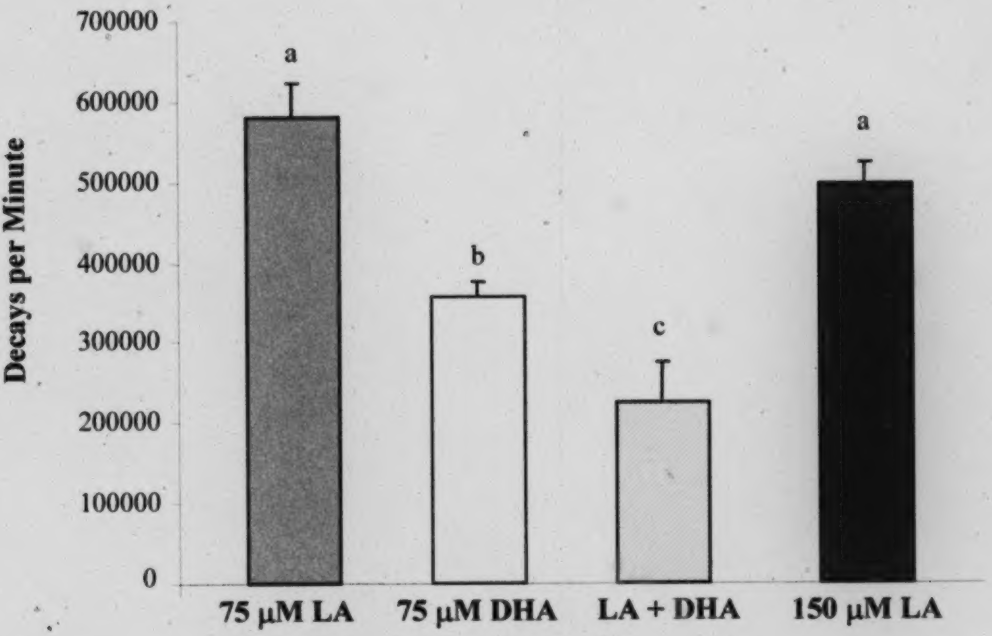

**FIGURE 7.2 Proliferation of MDA-MB-231 Breast Cancer Cells After 72 h Incubation With Fatty Acids.** Cells were seeded (2 x $10^4$ cell/mL/well) in 24-well sterile, flat-bottom tissue culture plates in culture media containing fetal calf serum (50 g/L) and allowed to adhere. After 48 h, the standard cell culture media was replaced with fresh media containing fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid) and cells were further incubated for 72 h. At 4 h prior to harvesting, cells were pulsed with [methyl-$^3$H]-thymidine as described in Materials and Methods. Bars represent means ± SEM (*n* = 5/treatment). Bars that do not share a common letter are significantly different ($p < 0.05$) as identified by a Duncan's multiple range test.

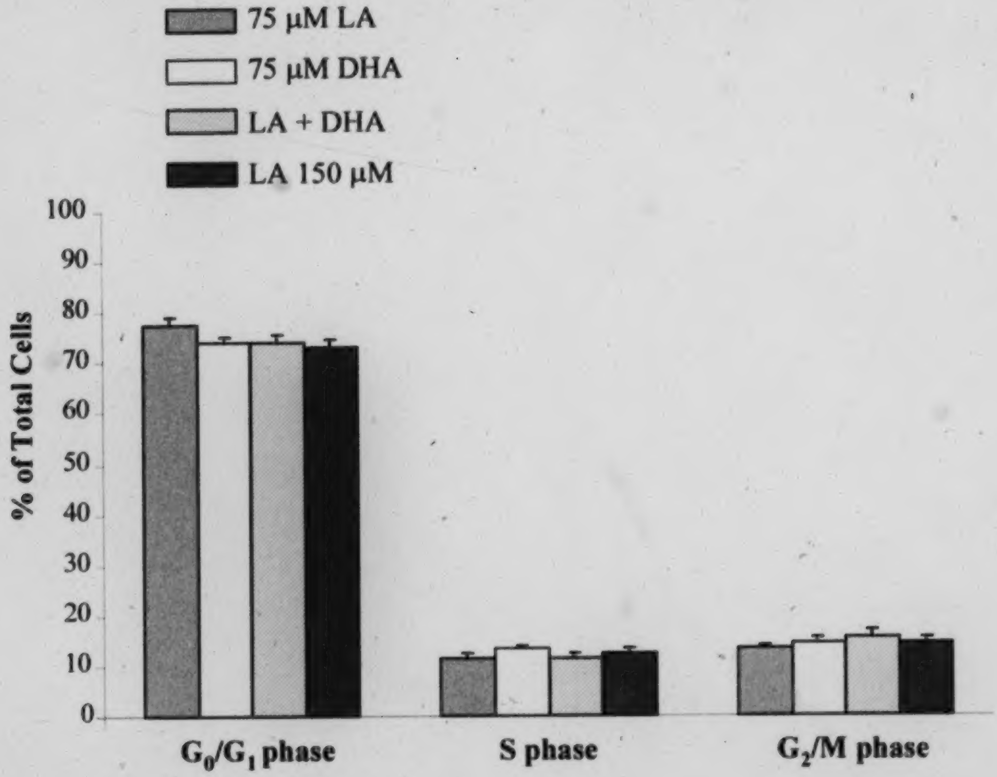

**FIGURE 7.3 Distribution of MDA-MB-231 Breast Cancer Cells Within the Cell Cycle Phases After 72 h Incubation With Fatty Acids.** MDA-MB-231 cells were incubated with fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid) for 72 h and DNA content was analyzed by propidium iodide staining and flow cytometry as described in Materials and Methods. Bars represent means ± SEM (*n* = 5/treatment). There were no significant differences between fatty acid treatments.

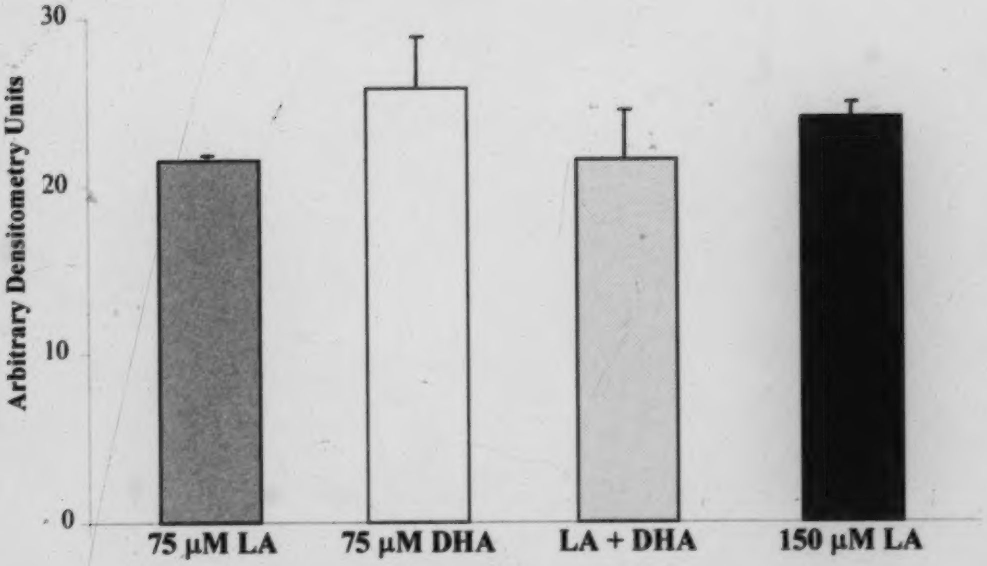

**FIGURE 7.4 Effect of 72 h Fatty Acid Incubation on Abundance of Proliferating-Cell Nuclear Antigen (PCNA) in MDA-MB-231 Human Breast Cancer Cells.** MDA-MB-231 cells were incubated with fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid) for 72 h and aliquots of cellular lystates (20 µg/lane) were examined by Western blot analysis using a monoclonal antibody to PCNA (PC10: *sc-56*) as described in Materials and Methods. Nitrocellulose membranes were stained with Ponceau S Solution to confirm equal protein loading per lane. Data represent the mean value (± SEM) of 2 experiments.

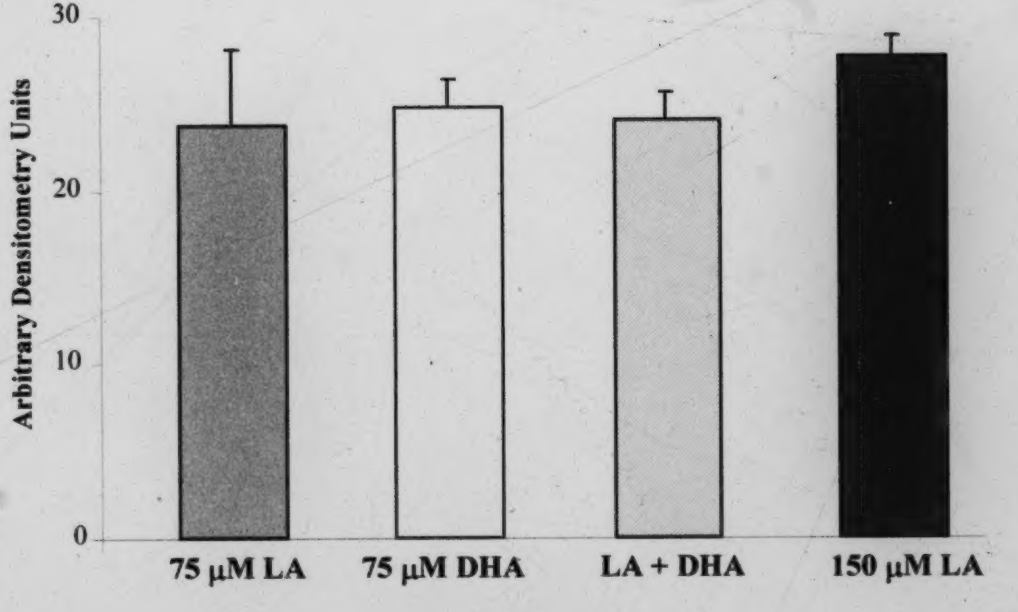

**FIGURE 7.5 Effect of 72 h Fatty Acid Incubation on Abundance of Proliferation-Related Kinase (PRK) in MDA-MB-231 Human Breast Cancer Cells.** MDA-MB-231 cells were incubated with fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid) for 72 h and aliquots of cellular lystates (20 μg/lane) were examined by Western blot analysis using a monoclonal antibody to PRK (B37-2) as described in Materials and Methods. Nitrocellulose membranes were stained with Ponceau S Solution to confirm equal protein loading per lane. Data represent the mean value (± SEM) of 2 experiments.

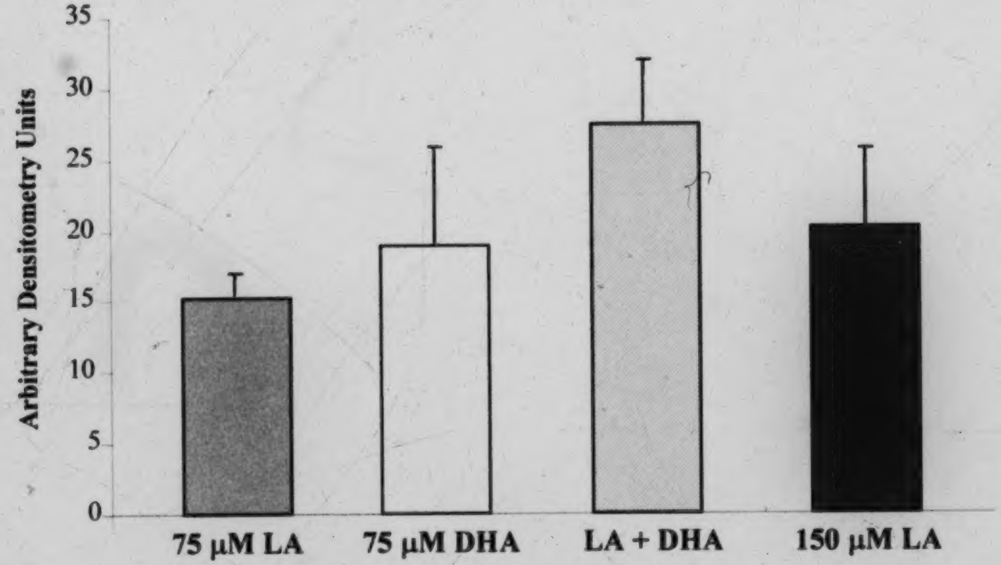

**FIGURE 7.6 Effect of 72 h Fatty Acid Incubation on Abundance of p53 in MDA-MB-231 Human Breast Cancer Cells.** MDA-MB-231 cells were incubated with fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid) for 72 h and aliquots of cellular lystates (20 μg/lane) were examined by Western blot analysis using a monoclonal antibody to p53 (DO-7) as described in Materials and Methods. Nitrocellulose membranes were stained with Ponceau S Solution to confirm equal protein loading per lane. Data represent the mean value (± SEM) of 2 experiments.

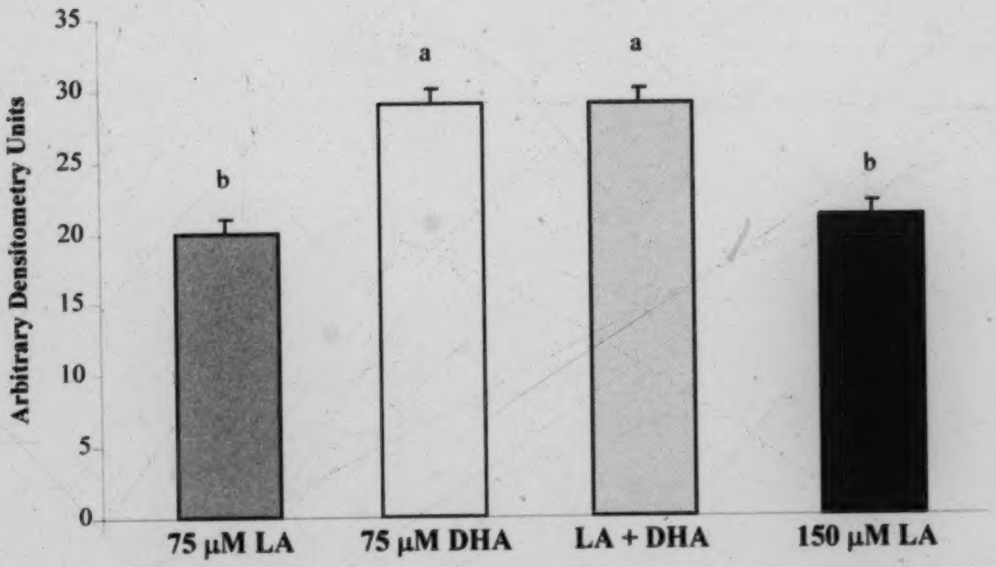

**FIGURE 7.7 Effect of 72 h Fatty Acid Incubation on Abundance of Retinoblastoma Protein in MDA-MB-231 Human Breast Cancer Cells.** MDA-MB-231 cells were incubated with fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid) for 72 h and aliquots of cellular lystates (20 μg/lane) were examined by Western blot analysis using a monoclonal antibody to Rb (G3-245) as described in Materials and Methods. Nitrocellulose membranes were stained with Ponceau S Solution to confirm equal protein loading per lane. Data represent the mean value (± SEM) of 2 experiments. Bars with different letters are significantly different ($p < 0.05$) as determined by a Duncan's multiple range test.

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# 8. AMINO ACID NUTRITION AND IMMUNE FUNCTION IN TUMOUR-BEARING RATS: A COMPARISON OF GLUTAMINE-, ARGININE- AND ORNITHINE α–KETOGLUTARATE-SUPPLEMENTED DIETS[1,2]

## 8.1 INTRODUCTION

Anticancer immunity consists, in part, of a cellular component involving macrophages and natural killer (NK) cells (Whiteside and Herberman 1995, Adams et al. 1982). There are many putative mechanisms of cytotoxicity by immune cells, including production of interleukin-2 (IL-2) by $CD4^+$ Th1 cells which activates both macrophages (Taub and Cox 1995) and NK cells (Whiteside and Herberman 1995). Interferon-gamma (IFN-γ), tumour necrosis factor-α (TNF-α) and nitric oxide (NO), produced by various activated immune cells, have also been identified as key molecules in anticancer defense (Cifone et al. 1995, Sotomayor et al. 1995, 1993). Since anticancer immunity declines progressively with tumour growth (Shewchuk et al. 1996), an important clinical goal is identifying means of stimulating host immunity. Recently, pharmaconutrients such as glutamine (Gln), arginine (Arg) and ornithine α-ketoglutarate (OKG) have attracted attention for their potential immunoenhancing properties.

Since Gln is an essential fuel for rapidly dividing immune cells (Wu et al. 1992), Gln-depleted states are associated with impaired immune function (Yaqoob and Calder 1997). Studies of Gln supplementation in tumour-bearing animals suggest improvement of anticancer immune defenses of T (Shewchuk et al. 1997), NK (Fahr et al. 1994) and lymphokine-activated killer cells (Juretic et al. 1994). Although Gln is the primary amino acid utilized by most rapidly proliferating tumours (Kovacevic and Morris 1972), Gln supplementation has been shown to have no effect or to inhibit tumour growth in animals (Shewchuk et al. 1997, Austgen et al. 1992, Klimberg et al. 1990).

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[1] A version of this chapter has been published. Robinson, L. E., Bussiere, F. I., Le Boucher, J., Farges, M.-C., Cynober, L .A., Field, C. J. & Baracos, V. E. (1999) Clinical Science 97: 657-669.

[2] This work was presented in part at Canadian Federation of Biological Societies, June 1998, Edmonton, Alberta and published in part in abstract form (L.E. Robinson, F.I. Bussiere, J. Le Boucher, L.A. Cynober, C.J. Field and V.E. Baracos. Dietary ornithine α-ketoglutarate is a more potent stimulator of anticancer immunity than glutamine or arginine).

An Arg requirement for optimal lymphocyte proliferation, T cell and macrophage function, and cytokine and NO production has been reported (Cynober et al. 1995a). Arg has been demonstrated to decrease growth of experimental tumours (Reynolds et al. 1990, Tachibana et al. 1985), an effect which may be mediated through the immune system (Brittenden et al. 1994). However, supplemental Arg can also augment tumour growth in some animal models and human tumours (Edwards et al. 1997, Grossie 1996, Park et al. 1992, Yeatman et al. 1991).

OKG has been proposed in the treatment of surgery, burns, sepsis, and cancer (Cynober 1995b). Potential mechanisms of action for OKG include synthesis of several key metabolites that become depleted in stress situations, including Gln, Arg and proline (Cynober 1995b). Since OKG may be metabolized to form Gln, Arg and polyamines, it clearly has potential as an immunoenhancing nutrient (Roch-Arveiller et al. 1996, Cynober 1995b, Albina 1993). Enterally fed OKG has been shown to have no effect on tumour growth in rats (Le Bricon et al. 1995, 1994).

In situations of stress, including cancer, trauma, burns, and sepsis, amino acids previously considered to be nonessential may become indispensable (Cynober 1995b). The demonstrated requirements for Arg, Gln and their precursors/metabolites, ornithine and α-ketoglutarate, are related, at least in part, to their involvement in cell proliferation and immune responses. In theory, these nutrients are metabolically interconvertible by known pathways, each potentially being a biosynthetic precursor of the others. Since it is not clear which of Arg, Gln, and OKG is most critical to antitumour defense and since they have not been compared systematically with one another in an internally controlled study, their relative efficacy is difficult to estimate. An additional difficulty in interpreting the current literature is that dietary amino acid supplementation has been studied over a wide range of cancer models and tumour burdens ranging in size from < 1 to 30% of host body weight (e.g. Shewchuk et al. 1997, Grossie 1996, Le Bricon et al. 1995, Yoshida et al. 1995, Reynolds et al. 1988). The following study was designed to compare the relative efficacy of dietary Gln, Arg and OKG to alter plasma and tissue amino acid profiles, tumour growth and host immune defense in rats bearing a tumour burden equivalent to ~ 0.8% of body weight and showing depletion of intracellular Gln, Arg and ornithine pools.

## 8.2 MATERIALS AND METHODS

**Animals and dietary design.** Animal studies were reviewed and approved by the Faculty of Agriculture, Forestry and Home Economics Animal Policy and Welfare Committee and were conducted in accordance with the Canadian Council on Animal Care guidelines. Female rats of the Buffalo strain were obtained from a colony maintained at the University of Alberta and were housed in individual cages, in a temperature (24 $^{O}$C) and humidity (80%) controlled room. After a 7 d adaptation period, rats were randomly allocated to serve as healthy controls (*n* = 16) or were implanted with the Morris Hepatoma 7777 (MH 7777, *n* = 64). MH 7777 is maintained as frozen stocks and is propagated by serial transplantation in rats of the same age, sex, and strain as those used in experiments. Briefly, rats were anesthetized with halothane and implanted subcutaneously in one flank with 25 μL of finely chopped MH7777 cells from a single donor animal (Le Bricon et al. 1995). Rats were randomly allocated to 5 treatments: 1) healthy controls fed a control mixture of amino acids, 2) tumour-bearing rats fed a control mixture of amino acids, 3) tumour-bearing rats fed a diet supplemented with Arg, 4) tumour-bearing rats fed a diet supplemented with Gln and 5) tumour-bearing rats fed a diet supplemented with OKG (**Table 8.1**). All diets met or exceeded the requirements of growing rats as specified by the National Research Council (1978). Semi-purified diets were formulated and prepared in the laboratory to be isoenergetic (gross energy, 15.48 MJ/kg diet) and isonitrogenous (26.1% crude protein, inclusive of the amino acid supplements described below). A constant portion of the diet (900 g/kg diet), was based on casein, cornstarch and dextrose, and contained 92% and 63% of the total dietary energy and protein, respectively. The constant portion of the diet also contained 200 g of fat from a mixture of sources (2% linseed oil, 39.2% hard beef tallow and 58.8% safflower oil), providing a polyunsaturated to saturated fatty acid ratio of ~ 0.9 (Chapter 3). A variable portion of the diet (100 g/kg diet) contained Gln (4.0% w/w), Arg (4.9% w/w), OKG (5.8% w/w) or a control mixture of amino acids. The control amino acid diet consisted of an isomolar mixture of four amino acids (alanine, glycine, serine and histidine) which have limited or no metabolic interaction with Gln, Arg and ornithine and are not known to be limiting for growth or immune function in rats of this age. The diets were isomolar for the N-containing constitutent (0.28 mol of Arg, Gln or ornithine) per kg of diet. The variable portion of each diet was made isonitrogenous and isoenergetic by the addition of the control amino acid mixture and/or cornstarch. From day 0 to day 14, rats consumed 2.7 ± 0.1, 3.2 ± 0.1 and 3.7 ± 0.2 g/kg/d of Gln, Arg and OKG, respectively. All rats were given free access to water and the experimental powder (not pelleted) diet for 14 d after tumour implantation. At the end of the study, rats were killed by $CO_2$ asphyxiation and cervical dislocation followed by collection of spleen, peritoneal macrophages and tibialis anterior muscle. In a subset of animals on each treatment ($n$ = 7/group), blood was collected under halothane anaesthesia by cardiac puncture for amino acid analysis. Due to the number of variables being measured, this study was carried out in a series of replicate experiments, such that all measurements were not performed on individual animals. For each assay, the number of animals in each treatment group is provided in the text, table or legend.

**Plasma and skeletal muscle amino acids.** Muscle and plasma free amino acid fractions were prepared as previously described (Le Bricon et al. 1994) and were separated by high performance liquid chromatography (HPLC) using a Varian 5000 HPLC (Varian, Palo Alto, CA) with a fluorichrom detector using ortho-phthaldehyde as the fluorescent reagent (Strelkov et al. 1989). The column used was a Supelcosil 3 μm LC-18 reverse phase column (4.6 x 150 mm, Supelco, Bellafonte, CA). Chromatographic peaks were recorded and integrated using a Shimadzu Ezchrom chromatography data system (Shimadzu Scientific Instruments, Columbia, MD).

**Immune variables.** A number of different tumour models in various inbred strains of rats are used for studying antitumour immune defense (Frey 1997). The MH 7777 is a well characterized, poorly differentiated and rapidly growing transplantable tumour that results in host death approximately 4 weeks after implantation, when the tumour has reached ~ 10% of body weight (Morris 1965). Tumour excision-rechallenge studies by Wepsic et al. (1976) demonstrated the presence of tumour antigens in the MH 7777 cell line. Previous work has demonstrated a progressive suppressive effect of this tumour on components of both natural and acquired antitumour immunity (Shewchuk et al. 1996).

In addition, the MH 7777 captures a significant amount of the amino acid intake of the host for protein synthesis and oxidiation (Le Bricon et al. 1995). Reduced tissue pools of Gln, glutamate and Arg during growth of the MH 7777 (Strelkov et al. 1989) may be associated with immune suppression. To measure antitumour immune defense, several well characterized *ex vivo* immune assays were used as substitues for *in vivo* immune assessment. Standard target cell lines (eg. YAC-1 or P815) are used extensively by immunological researchers to assess immune cell cytotoxicity or cytostasis in both humans and animals. Their use facilitates comparison of our results with those in the published literature.

**Peritoneal macrophage isolation and culture.** Resident peritoneal macrophages were obtained by sterile peritoneal lavage using cold RPMI 1640 (Gibco, Burlington, ON, Canada) containing glutamine (300 mg/L; Gibco) and 1% (v/v) antimycotic-antibiotic solution (0.1 IU/mL penicillin, 0.1 μg/mL streptomycin, 25 mg/L amphotericin B; Gibco), pH 7.4, and cell viability was assessed by trypan blue exclusion. Macrophages were washed by centrifugation at 380 g for 5 min at 4 °C and resuspended (4 x $10^9$ cells/L) in the appropriate volume of macrophage complete culture medium [RPMI 1640 containing 4% (v/v) heat-inactivated fetal calf serum (FCS, Gibco), glutamine (300 mg/L), 1% (v/v) antimycotic-antibiotic solution and 2-mercaptoethanol (2.5 μmol/L; Sigma Chemical, St. Louis, MO)], pH 7.4. Macrophages (0.8 x $10^6$/well) were incubated in sterile 96-well round bottom microtiter plates (Costar®, Corning Glass Works, Corning, NY) for 2 h in a humidified atmosphere at 37 °C in the presence of 5% $CO_2$ to allow for macrophage adherence. After 2 h, wells were washed 3 times in 200 μL of sterile Krebs' Ringer HEPES buffer (KRH, pH 7.4) at room temperature to remove non-adherent cells. The purity of the isolated macrophage population was assessed by indirect immunofluorescence assay as described below. Preliminary experiments showed that maximal stimulation of macrophages occurred using 10 μg/mL Lipopolysaccharride (LPS) from *Escherichia coli* (Serotype O55:B5; Sigma Chemical) for 24 h. Thus, for determination of NO and cytokine production, macrophages were incubated in complete culture medium with or without 10 μg/mL LPS. After 24 h, culture supernatants were collected and stored at –70 °C for subsequent NO and cytokine analyses as described below.

**Splenocyte isolation and activation.** Splenocytes were isolated aseptically in KRH (pH 7.4) supplemented with BSA (5 g/L; Sigma Chemical) as described in Chapter 3. Isolated splenocytes (3.0 x $10^9$ cells/L) in splenocyte complete culture medium (CCM; defined in Chapter 3) were incubated in sterile 24-well plates (Costar®) for 48 h in a humidified atmosphere at 37 °C in the presence of 5% $CO_2$. The cell culture medium contained either no mitogen (unstimulated cells) or was supplemented for 48 h with Concanavalin A (Con A) or phorbol myristate acetate (PMA) plus Ionomycin as described in Chapter 4.

**Indirect immunofluorescence (phenotype) assay.** Immune cell subsets in freshly isolated splenocytes and cultured splenocytes (unstimulated and Con A-stimulated) were identified by indirect immunofluorescence assay and flow cytometry as described in Chapter 3. Isolated peritoneal macrophages were also identified by this assay. All monoclonal antibodies (mAb) used were defined in Chapters 3 and 5 and are summarized in **Table 8.2**.

**Macrophage cytostatic activity**. Macrophage-sensitive P815 murine mastocytoma cells (American Type Culture Collection, Rockville, MD) were maintained in Dulbecco's minimal essential medium (Sigma Chemical) at 37 °C and 5% $CO_2$ and the medium was replaced every 2 d during the experiment. Prior to being used, P815 cells were washed by centrifugation at 380 g for 10 min at 4 °C, counted with a hemocytometer and resuspended in the appropriate volume of macrophage complete culture medium (described above) to obtain 0.2 x $10^9$ cells/L. Macrophage cytostatic activity was measured by a slightly modified version of the method of Lopez et al. (1996), based on the inhibition of [methyl-$^3$H]-thymidine incorporation by macrophage-sensitive P815 target cells in coculture with macrophages. P815 cells (4 x $10^4$) were added to varying amounts of macrophages to achieve effector:target cell ratios between 0:1 and 10:1. Immediately after adding the P815 cells, 0.5 μCi [methyl-$^3$H]-thymidine (Amersham, Oakville, ON, Canada) was added to each well. Preliminary experiments showed that a 6 h incubation of the co-culture at 37 °C and 5% $CO_2$ was sufficient to demonstrate inhibition of [methyl-$^3$H]-thymidine incorporation by P815 target cells. After 6 h, cells were harvested onto glass-fiber filters using a multi-well harvester (Skatron Instruments, Lier, Norway) to eliminate non-incorporated radioactivity. The dried filters were dissolved in 4 mL Ecolite® scintillation fluid (ICN, Montreal, QB, Canada) and the radioactivity incorporated into the P815 cells was measured using a Beckman beta counter (LS 5801; Beckman Instruments Inc., Mississauga, ON, Canada). Macrophage cytostatic activity was determined using the value obtained from incubating the target cells alone (ie. 0:1 ratio) as 100% and the following formula: Marcrophage cytostatic activity = 100 - (100 x cpm TM/cpm T) where cpm TM = radioactivity (counts per min) in target cell:macrophage coculture and cpm T = radioactivity in target cell culture.

**NK cell cytotoxicity assay.** A 4 h sodium chromate ($^{51}$Cr; Amersham, Oakville, ON, Canada) release assay was performed using NK cell sensitive murine lymphoma YAC-1 cells (American Type Culture Collection) as targets and freshly isolated splenocytes as effector cells in effector:target cell ratios from 25:1 to 100:1 as described in Chapter 3.

### Mechanisms of immune cell cytotoxicity and cytostasis

**Splenocyte IL-2 production.** IL-2 concentration in culture supernatants collected from unstimulated and stimulated splenocytes was determined by a colorimetric enzyme-linked immunosorbent assay (ELISA) as described in Chapter 4.

**Splenocyte IFN-γ and TNF-α production.** IFN-γ and TNF-α concentrations in supernatants collected from unstimulated and stimulated splenocytes were determined by ELISA as described in Chapter 6.

**NO production.** NO production was estimated by analyzing nitrite ($NO_2^-$) concentration in culture supernatants using a colorimetric assay based on the Griess reaction (Green et al. 1982) as described in Chapter 4. In some experiments, inhibitors of NO synthase activity, NG-nitro-L-arginine methyl ester (L-NAME, 1200 nmol/$10^6$ cells; Sigma Chemical) or S-methylisothiourea (SMT, 120 nmol/$10^6$ cells; Sigma Chemical), were added to the NK cell cytotoxicity and NO assays.

**Statistical analysis.** Results are presented as means ± SEM. All statistical analyses were conducted using the SAS statistical package (Version 6.11, SAS Institute, Cary, NC). For phenotype, cytotoxicity, NO, cytokine, and plasma and tissue amino acid data, the effect of the tumour in rats fed the control amino acid diet was determined by one-way analysis of variance (ANOVA). Similarly, for tumour-bearing rats the effect of diet was analyzed by one-way ANOVA. Least-squares means were used to identify significant ($p < 0.05$) differences among treatment groups. Food intake, cytostatic activity and cytotoxicity data were also compared among groups by one-way split-plot (repeated measures) ANOVA (Steele and Torrie 1980). Paired *t* tests were used to compare cytokine and $NO_2^-$ production by immune cells with or without mitogen.

## 8.3 RESULTS

**Food intake and body, tumour and spleen weights.** There were no treatment differences in food intake (**Figure 8.1**). However, food intake (on a g per kg body weight basis) of both healthy and tumour-bearing rats started to decline at day 12 ($p < 0.05$, by repeated measures ANOVA, Figure 8.1). Initial and final body weights and tumour weight in the tumour-bearing rats (0.77 ± 0.08% body weight, $n = 30$) were not significantly different among groups (**Table 8.3**). Spleen weight and the number of spleen cells isolated per gram of spleen did not differ between healthy and tumour-bearing rats fed the control diet (Table 8.3). There was no significant difference in spleen weight among tumour-bearing rats fed the control, Arg- or OKG-supplemented diets (Table 8.3). However, spleen weight in tumour-bearing rats fed the Gln-supplemented diet was increased (+12%, $p < 0.04$) compared with tumour-bearing rats fed the control diet (Table 8.3). For tumour-bearing rats, diet did not significantly affect the number of spleen cells isolated per gram of spleen (Table 8.3).

**Biochemical and immunological analysis**

**Plasma and skeletal muscle amino acid concentrations.** There were no significant effects of the tumour or diet on plasma amino acid concentrations. However, tibialis anterior muscles isolated from tumour-bearing animals fed the control diet showed significant reductions in free glutamate (-54%), Gln (-30%) and Arg (-25%) compared with healthy control rats (**Table 8.4**). The largest effect of the diet treatments was on these amino acids in muscles of tumour-bearing rats (Table 8.4). Dietary supplementation with Gln and OKG increased muscle Gln concentrations (+52% and +56%, respectively, $p < 0.05$ compared with tumour control), while Arg elicited a similar, but nonsignificant effect (Table 8.4). There was not, however, a complete restoration of muscle Gln concentration by any diet treatment (Table 8.4). Supplemental Arg and OKG treatments raised muscle Arg concentrations to levels seen in healthy animals, while dietary Gln did not have this effect (Table 8.4).

**Immune cell phenotypes.** The tumour did not significantly affect the proportion of immune cell phenotypes in freshly isolated splenocytes with the following exception; freshly isolated splenocytes from tumour-bearing rats fed the control diet had a lower (-21%; $p < 0.04$) proportion (% of total) of macrophages and a higher (+13%; $p < 0.03$) proportion of CD4$^+$ T helper cells expressing CD28 compared with healthy rats (**Table 8.5**). The relative proportion of freshly isolated splenocytes expressing the IL-2 receptor (CD25) was negligible ($\leq$ 1%) and was not significantly different between control and tumour-bearing rats fed the control diet (Table 8.5). Although 48 h splenocyte stimulation with Con A increased CD25 expression, the relative proportion of immune cell phenotypes expressing CD25 after stimulation was not significantly affected by the presence of a tumour (**Table 8.6**).

For tumour-bearing rats, diet did not significantly affect immune cell phenotypes in freshly isolated cells with the following exception: the relative proportion of macrophages was higher (+23%, $p < 0.01$) from tumour-bearing rats fed the Gln-supplemented diet compared with those fed the control diet, but not the other diets (Table 8.5). The relative proportion of freshly isolated splenocytes from tumour-bearing rats expressing CD25 was negligible and was not significantly affected by diet (1.0 ± 0.1%, *n* = 29, Table 8.5). For tumour-bearing rats, diet did not significantly affect the relative proportion of $CD25^+$ cells identified as $CD4^+$ T helper cells, $CD8^+$ T suppressor/cytotoxic cells, B cells, macrophages or $CD28^+$ cells after Con A stimulation (Table 8.6). However, after Con A stimulation, tumour-bearing rats fed the OKG-supplemented diet had a higher ($p < 0.05$) proportion of $CD25^+$ $CD5^+$ T cells compared with rats fed the control or Arg-supplemented diet, but not the Gln-supplemented diet (Table 8.6).

**Macrophage cytostatic activity.** The purity of the peritoneal macrophage preparations obtained by sterile peritoneal lavage was assessed by the OX19 and OX42 mAb. The proportion of T cells in the peritoneal cell preparations (assessed by OX19) was negligible (1.3 ± 0.3%, *n* = 16), whereas the proportion of macrophages (assessed by OX42) was 81 ± 4% (*n* = 16). Neither tumour nor diet significantly affected the proportion of $OX42^+$ cells obtained by peritoneal lavage. Macrophage cytostatic activity against P815 cells was lower (-30%, $p < 0.05$) for tumour-bearing rats fed the control diet compared with healthy control rats as determined by one-way split-plot (repeated measures) ANOVA (**Figure 8.2**). A significant effect of diet was also determined by one-way split-plot (repeated measures) ANOVA; tumour-bearing rats fed the OKG-supplemented diet had higher (+39%, $p < 0.05$) macrophage cytostatic activity compared with tumour-bearing rats fed the control diet, but not the Gln- or Arg-supplemented diets (Figure 8.2).

**NK cell cytotoxicity.** NK cell cytotoxicity data was analyzed by one-way split-plot (repeated measures) ANOVA (**Figure 8.3**). NK cell cytotoxic activity was not significantly different between healthy and tumour-bearing rats fed the control diet. Similarly, NK cell cytotoxicity was not significantly different among tumour-bearing rats fed the control, Arg- or Gln-supplemented diets as determined by split-plot (repeated measures) ANOVA. However, tumour-bearing rats fed the OKG-supplemented diet had increased NK cell cytotoxic activity ($p < 0.01$) compared with tumour-bearing rats fed the other diets. Results were also calculated as lytic units with one lytic unit being equal to the number of effector cells ($\times 10^{-3}$) required to cause 20% lysis of target cells. Tumour-bearing rats fed the control diet had lower lytic units compared with healthy rats fed the same diet (39 ± 3 compared with 49 ± 3, respectively, $n \geq 7$/diet, $p < 0.02$, **Figure 8.4**). For tumour-bearing rats, lytic units were not significantly different among diet groups (40 ± 2, $n = 27$, Figure 8.4).

### Mechanisms of immune cell cytotoxicity and cytostasis

**Splenocyte cytokine production.** IL-2, IFN-γ, and TNF-α production was higher from splenocytes stimulated with Con A or PMA plus Ionomycin for 48 h compared with unstimulated cells regardless of treatment or diet ($p < 0.05$, by paired *t* test, unstimulated data not illustrated). Neither the presence of a tumour nor diet significantly affected the amount of IL-2, IFN-γ and TNF-α produced by mitogen-stimulated splenocytes (**Table 8.7**).

**NO production.**

***(a) Splenocytes.*** Neither tumour nor diet significantly affected the amount of $NO_2^-$ produced by unstimulated splenocytes (**Figure 8.5**). For both healthy and tumour-bearing rats fed the control diet, $NO_2^-$ production was higher ($p < 0.05$) from splenocytes stimulated with Con A or PMA plus Ionomycin for 48 h compared with unstimulated cells (Figure 8.5, statistics not illustrated). For tumour-bearing rats fed either the Gln-, Arg- or OKG-supplemented diets there was no significant effect of Con A stimulation on splenocyte $NO_2^-$ production (Figure 8.5, statistics not illustrated). However, for all diet groups, $NO_2^-$ production was higher ($p < 0.003$) from splenocytes stimulated with PMA plus Ionomycin compared with unstimulated cells (Figure 8.5, statistics not illustrated). The presence of a tumour did not significantly affect $NO_2^-$ production by mitogen-stimulated splenocytes when rats were fed the control diet (Figure 8.5). Following Con A stimulation, splenocytes from tumour-bearing rats fed the OKG-supplemented diet produced higher concentrations of $NO_2^-$ ($p < 0.02$) compared with splenocytes from tumour-bearing rats fed either the control diet or Arg-supplemented diet (Figure 8.5). Following PMA plus Ionomycin stimulation, splenocytes from tumour-bearing rats fed the OKG-supplemented diet produced higher amounts of $NO_2^-$ (+28%; $p < 0.04$) compared with tumour-bearing rats fed the control diet (Figure 8.5).

L-NAME and SMT inhibited splenocyte $NO_2^-$ production for up to 48 h (**Figure 8.6**). L-NAME also inhibited ($p < 0.05$) NK cell cytotoxicity consistently in splenocytes isolated from tumour-bearing rats fed either the control, Arg- or OKG-supplemented diets (**Figure 8.7**). This assay was not performed on splenocytes from the Gln-supplemented group. SMT did not inhibit NK cell cytotoxicity in any of the diet groups (Figure 8.7).

***(b) Macrophages.*** In all groups, except the Gln-supplemented diet group, $NO_2^-$ production was higher from macrophages stimulated with LPS compared with unstimulated cells ($p < 0.05$, by paired *t* test, statistics not illustrated). The tumour did not significantly affect the amount of $NO_2^-$ produced by unstimulated or LPS-stimulated macrophages when rats were fed the control diet (**Figure 8.8**). Both unstimulated and LPS-stimulated macrophages from tumour-bearing rats fed the OKG-supplemented diet produced higher ($p < 0.05$) concentrations of $NO_2^-$ compared with macrophages from tumour-bearing rats fed the Arg-supplemented diet (Figure 8.8).

## 8.4 DISCUSSION

### Nutritional and immune status of tumour-bearing animals

Prior studies have examined animals during progressive growth of the MH 7777 (Shewchuk et al. 1996, Le Bricon et al. 1995, Strelkov et al. 1989). At two weeks post-implantation, tumours attain ~ 0.8% of body mass and food intake and N balance begin to decline. During the third week, when there is a strong deterioration of food intake and N balance, changes in body weight are almost entirely attributable to a rapid increase in tumour mass. Together, the rate of protein deposition and amino acid oxidation by the tumour may attain in excess of 70% of daily protein intake by the host during the third week after implantation (Le Bricon et al. 1995). These results suggest that the nutritional status of animals bearing tumours can change from moderate to poor to catastrophic in a short period of time. Thus, host nutritional status must be carefully defined under the experimental conditions used so as to clearly distinguish the effects of altered nutritional status on measured variables. Dietary supplementation with Gln, Arg and OKG has been studied over a wide range of tumour types and burdens (e.g. Edwards et al. 1997, Shewchuk et al. 1997, Grossie 1996, Klimberg et al. 1996, Le Bricon et al. 1995, 1994, Yoshida et al. 1995, Fahr et al. 1994, Kaibara et al. 1994, Oka et al. 1994, Austgen et al. 1992, Yeatman et al. 1991). Results from these studies are near impossible to compare, at least in part, due to vast differences in nutritional status of the animals studied. In the present study, a defined, limited stage of cancer associated with lowered amino acid levels in skeletal muscle pools (Strelkov et al. 1989) was selected for comparison of Gln, Arg and OKG supplementation.

Of the immune indices measured, the major changes elicited by the tumour-bearing state were evident in macrophages. Specifically, both the proportion of macrophages in spleen (Table 8.5) and macrophage cytostatic activity (Figure 8.2) were reduced at two weeks after tumour implantation; the combination of these effects is a substantial reduction in overall macrophage antitumour defense. Suppression of macrophage cytostatic activity during progressive tumour growth has been demonstrated in other models (Sotomayor et al. 1995). One potential mechanism may be a reduced capacity of macrophages from tumour-bearing hosts to produce NO (Sotomayor et al. 1995), a finding which has been associated with diminished inducible NO synthase expression (DiNapoli et al. 1996). In the present study, production of NO by peritoneal macrophages was not affected by the tumour (Figure 8.8). However, it cannot be concluded from this study whether other mechanisms, such as the production of NO by tumour-infiltrating macrophages or the production of prostaglandin $E_2$ (Klimberg et al. 1996) are involved in suppression of macrophage activity in this tumour model.

## Diets and immune function

Recent investigation has focused on supplementing enteral or total parenteral nutrition with various immunoenhancing nutrients, including amino acids (Roch-Arveiller et al. 1996, Shewchuk et al. 1996), either alone or as part of multiple nutrient formulations (Braga et al. 1999, Atkinson et al. 1998, Kemen et al. 1995). Unfortunately, the design of most studies has been such that it is not possible to draw conclusions as to the efficacy of individual nutrients for improving immunity. The present study is the first to compare the relative efficacy of Gln, Arg and OKG to improve immune defense in tumour-bearing rats. Overall, the effect of diet on the immune indices measured was limited. In particular, Gln and Arg had minimal effects on spleen cell phenotype distribution (Table 8.5) and did not induce significant alterations in macrophage or NK cell cytotoxicity (Figures 8.2-8.3), NO (Figures 8.5 and 8.8) or cytokine production (Table 8.7). By contrast, OKG appeared to enhance certain immune functions, albeit to a limited extent. Tumour-bearing rats fed the OKG-supplemented diet had increased (+22%) NK cell cytotoxicity (Figure 8.3) but no change in the proportion of NK cells in spleen (Table 8.5), suggesting that OKG enhanced the function of NK cells. Tumour-bearing rats fed the OKG-supplemented diet had higher (+39%) macrophage cytotostatic activity (Figure 8.2), indicating a general enhancing effect of OKG on cell-mediated cytotoxic capacity. In effect, feeding tumour-bearing rats OKG counteracted the reduction in macrophage cytostatic activity seen at two weeks after tumour implantation. Our results are in agreement with those of Albina (1993) who showed that feeding healthy rats OKG (6.15 g/kg/d) increased macrophage cytotoxicity *in vitro*. There are multiple putative effectors of macrophage and NK cell cytotoxicity. We attempted to explain observed diet-induced differences in cytotoxicity in terms of immune cell production of cytokines and NO, compounds which may be factors in upregulation of immune cell cytotoxicity (Whiteside and Herberman 1995, Ortaldo et al. 1984). Tumour-bearing rats fed the OKG-supplemented diet had a higher (+10%) proportion of total T cells expressing the IL-2 receptor ($CD25^+$) after mitogen stimulation (Table 8.6). However, diet did not significantly affect splenocyte IL-2, IFN-γ or TNF-α production (Table 8.7), suggesting that diet-induced differences in cytotoxicity are not mediated by these cytokines. Tumour-bearing rats fed the OKG-supplemented diet also had higher splenocyte NO production (Figure 8.5). To investigate NO as a potential mediator of NK cell cytotoxicity we used two inhibitors of NO synthase. SMT, a specific inhibitor of the inducible NO synthase, did not inhibit NK cell cytotoxicity (Figure 8.7) even though it was effective in suppressing splenocyte nitrite production (Figure 8.6). Incubations with L-NAME, a nonspecific NO synthase inhibitor, showed a reproducible inhibition (~15%) of cytotoxicity (Figure 8.7) which is consistent with those in the literature (Filep et al. 1996). However, the fact that SMT is ineffective in inhibiting NK cell cytotoxicity and suggestions that many of the NO synthase inhibitors, including L-NAME, may exert nonspecific effects through interactions with other iron-containing enzymes (Peterson et al. 1992), imply that NK cell cytotoxicity against YAC-1 cells is not mediated by NO. Thus, we are presently lacking a complete description of immune cell cytotoxicity mechanisms and the role of amino acids therein. While this awaits further study, the possible role of OKG in the generation of polyamines (Vaubourdolle et al. 1990) as well as the role of OKG in reducing hyperammonemia (Molinard et al. 1982) should be investigated as alternate mechanisms in relation to anticancer immune defense.

### Diets and tumour growth

Feeding a nutritionally complete, semi-purified diet supplemented with either Gln, Arg, or OKG had no net effect on growth of the MH 7777 compared with a control mixture of nonessential amino acids (Table 8.3). Animals bearing a tumour showed specific depletion of intracellular Gln, glutamate and Arg pools in skeletal muscle (Table 8.4) and these were temporarily associated with reductions in macrophage numbers (Table 8.5) and cytostatic activity (Figure 8.2). Diet treatments were associated with positive changes in muscle free Gln, glutamate and Arg pools (Table 8.4), but only OKG was associated with a significant improvement in immune variables in tumour-bearing animals. Overall, the results suggest no net advantage for antitumour immunity of these diet treatments in this model. It has been proposed that when tumour cells are highly susceptible to recognition and destruction by the host immune system, then the ability of Arg to upregulate antitumour defense can result in a decrease in tumour growth (Reynolds et al. 1988). Since the MH is modestly immunogenic (Wepsic et al. 1976), a logical next step would be to compare dietary Gln, Arg and OKG in tumours expressing a greater degree of immunogenicity. Such a systematic approach would begin to lend understanding to a literature which is presently very difficult to interpret. There are currently at least 30 published papers focusing on Gln, Arg and OKG supplementation and tumour growth. In the Gln literature, some studies have found no effect of Gln on tumour growth (e.g. Yoshida et al. 1995, Kaibara et al. 1994, Austgen et al. 1992, Klimberg et al. 1990), while others have shown tumour growth inhibition by supplemental Gln (e.g. Shewchuk et al. 1997, Klimberg et al. 1996, Fahr et al. 1994). Arg supplementation has been reported to have either no effect on tumour growth (e.g.

Oka et al. 1994), to stimulate (e.g. Edwards et al. 1997, Grossie 1996, Yeatman et al. 1991) or to inhibit tumour growth (e.g. Reynolds et al. 1990, 1988, Tachibana et al. 1985). In two reports to date, dietary OKG has not affected tumour growth (Le Bricon et al. 1995, 1994). Individual studies have used a wide variety of tumour types and hosts, tumour immunogenicity, both enteral and parenteral feeding regimens, levels of amino acid supplementation ranging from 1 to 10% w/w of the diet, tumour burdens ranging from < 1 to 30% of host body weight and a variety of "control diets". Given this diversity, there is in most cases no obvious explanation as to why specific amino acids might increase or decrease tumour growth in different experimental situations. At present we have no explanation as to why Gln inhibited tumour growth in a prior experiment in our laboratory (Shewchuk et al. 1997). It may be that a number of variables, for which we currently cannot account, impact upon the balance between stimulation and inhibition of tumour growth by dietary amino acid supplementation.

Further understanding of the roles of individual amino acids in specific functions of immune and tumour cells would generate clarification of the immunomodulatory potential of amino acids in cancer. The roles of amino acids may be complex. For example, the NO-producing breast cancer cell line (EMT-6) studied by Edwards et al. (1997) is potently stimulated by Arg supplementation. If antitumour defenses are also susceptible to Arg supply either through its role as a NO precursor or other mechanisms, then it is clearly the balance of power between these pro- and anti-tumour effects which becomes of importance. In the absence of differences in tumour growth, the potential importance of diet-associated changes in the measured immune variables is not clear. However, the ability of specific nutrients to enhance the immune system should not be overlooked, since improved host immunity may be beneficial in terms of prevention of disease recurrence, metastasis, or infectious complications, and may be important in host tolerance to immunosuppressive treatments such as chemotherapy. Our results do not preclude such benefits, however these must be tested in separate experiments.

**TABLE 8.1 Composition of Experimental Diets Fed to Rats[1]**

| **Constant Portion** 900 g/kg diet | | **Variable Portion** 100g/kg diet | | | | |
|---|---|---|---|---|---|---|
| | | | ***Diet*** | | | |
| | | | **Control** | **Gln** | **Arg** | **OKG** |
| ***Ingredient*** | g/kg diet | ***Ingredient*** | g/kg diet | | | |
| Casein | 166 | Cornstarch | 20 | 19 | 51 | 1 |
| Cornstarch | 205 | Control amino acid mixture | 80 | 41 | -- | 41 |
| Glucose | 210 | Arg | -- | -- | 49 | -- |
| Cellulose | 50 | Gln | -- | 40 | -- | -- |
| Fat | 200 | OKG | -- | -- | -- | 58 |
| L-methionine | 2 | **g N / 100 g** | 15.8 | 15.8 | 15.8 | 15.8 |
| Vitamin * | 10 | | | | | |
| Mineral * | 50 | | | | | |
| Choline | 1 | | | | | |
| Inositol | 6 | | | | | |
| **Total** | 900 | **Total** | 100 | 100 | 100 | 100 |

[1] Diets were isoenergetic (gross energy, 15.48 MJ/kg diet) and isonitrogenous (26.1 % crude protein, inclusive of the amino acid supplements). The glutamine (Gln), arginine (Arg) and ornithine α-ketoglutarate (OKG) supplemented diets contained 0.28 mol/kg diet of Gln, Arg and ornithine, respectively. The control amino acid mixture was an isomolar mixture of alanine, glycine, serine and histidine. The fat mixture contained 2% linseed oil, 39.2% hard beef tallow, and 58.8% safflower oil, providing a polyunsaturated to saturated fatty acid ratio of ~ 0.9 (as described in Chapter 3). All rats were given free access to the experimental diet and water for 14 d after tumour implantation.

* AIN76, ICN Biochemicals, Cleveland, OH (American Institute of Nutrition 1977).

**TABLE 8.2 Monoclonal Antibodies Used in Indirect Immunofluorescence (Phenotype) Assay**

| Clone[1] | Specificity | Description |
|---|---|---|
| OX19 | CD5 | Reacts with 69 kDa surface glycoprotein on thymocytes and peripheral T cells |
| w3/25 | CD4 | Reacts with 48-53 kDa surface glycoprotein on T helper cells and peritoneal macrophages |
| OX8 | CD8a | Reacts with 34 kDa α chain of CD8 antigen on thymocytes, T suppressor/cytotoxic cells, natural killer (NK) cells, and some activated T helper cells |
| OX12 | Ig κ chain | Reacts with immunoglobulin (Ig) κ chain on B cells |
| OX42 | CD11b/c | Reacts with 160, 103, and 95 kDa cell surface proteins on most resident peritoneal and activated macrophages, granulocytes, monocytes, and dendritic cells |
| 3.2.3 | CD161 | Reacts with NKR-P1A on NK cells and subset of T cells |
| JJ319 | CD28 | Reacts with a costimulatory receptor for activation on T cells and a subset of NK cells |
| OX39 | CD25 | Reacts with the α-chain of the interleukin-2 receptor on activated T and B cells |

[1] All monoclonal antibodies were kindly provided by Dr. A. Rabinovitch (University of Alberta, Edmonton, AB, Canada) with the exceptions of 3.2.3 and OX39 (Cedarlane Laboratories Ltd., Hornby, ON, Canada) and JJ319 (PharMingen, Mississauga, ON, Canada). All mAb were mouse anti-rat IgG.

TABLE 8.3 Body, Tumour and Spleen Weights of Rats[1]

| *Animal treatment*<br>*Diet* | **Healthy Control** | **Tumour Control** | **Tumour Gln** | **Tumour Arg** | **Tumour OKG** |
|---|---|---|---|---|---|
| Initial Body Weight, g | 188 ± 8 | 198 ± 10 | 192 ± 9 | 196 ± 5 | 194 ± 10 |
| Final Body Weight, g [2] | 203 ± 6 | 212 ± 11 | 209 ± 8 | 213 ± 5 | 213 ± 6 |
| Tumour Weight, g | -- | 1.4 ± 0.2 | 1.8 ± 0.5 | 1.5 ± 0.4 | 1.7 ± 0.4 |
| Spleen Weight, mg/100 g body weight | 203 ± 5 | 216 ± 8 | 241 ± 8 * | 228 ± 5 | 225 ± 7 |
| Spleen Cells/g spleen ($10^6$) | 380 ± 24 | 409 ± 23 | 369 ± 12 | 378 ± 31 | 351 ± 18 |

[1] Buffalo rats were fed semi-purified diets supplemented with glutamine (Gln), arginine (Arg), ornithine α-ketoglutarate (OKG) or a control mixture of nonessential amino acids (Control) for 14 d post-implantation of the Morris Hepatoma 7777. Healthy rats were not implanted with a tumour and were fed a control mixture of nonessential amino acids in the diet. Values are means ± SEM ($n \geq 7$/group).

[2] Final body weight includes tumour weight.

* Significantly different from tumour-bearing rats fed the control diet ($p < 0.04$, by ANOVA and least squares means).

**TABLE 8.4 Free Amino Acid Concentrations of Tibialis Anterior Muscle of Rats**[1]

| *Animal treatment*<br>*Diet* | **Healthy**<br>**Control** | **Tumour**<br>**Control** | **Tumour**<br>**Gln** | **Tumour**<br>**Arg** | **Tumour**<br>**OKG** |
|---|---|---|---|---|---|
| **Amino acid** | | | nmol/g tissue | | |
| Glutamate | $2.38 \pm 0.31^{a}$ | $0.93 \pm 0.07^{b}$ | $1.30 \pm 0.13^{c}$ | $1.09 \pm 0.09^{b}$ | $1.34 \pm 0.1^{c}$ |
| Glutamine | $4.65 \pm 0.32^{a}$ | $1.98 \pm 0.34^{b}$ | $3.01 \pm 0.26^{c}$ | $2.89 \pm 0.33^{bc}$ | $3.10 \pm 0.36^{c}$ |
| Arginine | $2.28 \pm 0.22^{a}$ | $1.72 \pm 0.08^{b}$ | $1.56 \pm 0.11^{b}$ | $2.37 \pm 0.28^{a}$ | $2.05 \pm 0.10^{a}$ |
| Ornithine | $0.17 \pm 0.02^{a}$ | $0.14 \pm 0.01^{a}$ | $0.13 \pm 0.01^{a}$ | $0.14 \pm 0.02^{a}$ | $0.12 \pm 0.01^{a}$ |

[1] Buffalo rats were fed semi-purified diets supplemented with glutamine (Gln), arginine (Arg), ornithine α-ketoglutarate (OKG) or a control mixture of nonessential amino acids (Control) for 14 d post-implantation of the Morris Hepatoma 7777. Healthy rats were not implanted with a tumour and were fed a control mixture of nonessential amino acids in the diet. Values are means ± SEM ($n \geq 7$/group). Means within a row that do not share a common superscript letter are significantly different ($p < 0.05$, by ANOVA and least squares means).

**TABLE 8.5 Immune Cell Phenotypes in Freshly Isolated Rat Splenocytes[1]**

| *Animal Treatment* | Healthy | Tumour | Tumour | Tumour | Tumour |
|---|---|---|---|---|---|
| *Diet* | **Control** | **Control** | **Gln** | **Arg** | **OKG** |
| **Immune Cell Phenotype** | | | % of total immune cells in spleen | | |
| $CD5^+$ T cells | 60 ± 1 | 61 ± 1 | 59 ± 1 | 60 ± 1 | 60 ± 1 |
| $CD5^+$ $CD4^+$ T helper cells | 32 ± 1 | 36 ± 1 | 34 ± 1 | 36 ± 1 | 34 ± 1 |
| $CD5^+$ $CD8^+$ T suppressor/cytotoxic cells | 12 ± 1 | 14 ± 1 | 14 ± 1 | 14 ± 1 | 13 ± 1 |
| CD4/CD8 ratio | 3.3 ± 0.5 | 2.8 ± 0.4 | 3.3 ± 0.3 | 2.8 ± 0.1 | 2.8 ± 0.1 |
| B cells | 23 ± 1 | 24 ± 1 | 25 ± 1 | 23 ± 1 | 24 ± 1 |
| Macrophages | 7.7 ± 0.5 | $6.1 \pm 0.2^{*,a}$ | $7.5 \pm 0.4^{b}$ | $7.1 \pm 0.5^{ab}$ | $7.3 \pm 0.4^{ab}$ |
| Natural killer cells | 7.3 ± 0.5 | 6.0 ± 0.7 | 6.5 ± 0.4 | 6.4 ± 0.6 | 6.8 ± 0.6 |
| $CD5^+$ $CD28^+$ cells | 59 ± 1 | 57 ± 1 | 56 ± 2 | 56 ± 1 | 58 ± 1 |
| $CD4^+$ $CD28^+$ cells | 32 ± 1 | $36 \pm 1^*$ | 35 ± 1 | 36 ± 2 | 33 ± 1 |
| $CD8^+$ $CD28^+$ cells | 15 ± 1 | 16 ± 1 | 15 ± 2 | 17 ± 1 | 17 ± 1 |
| $CD25^+$ cells | 1.1 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 | 1.0 ± 0.1 | 1.1 ± 0.1 |

[1] Splenocytes were isolated from healthy or tumour-bearing rats fed semi-purified diets supplemented with glutamine (Gln), arginine (Arg), ornithine α-ketoglutarate (OKG) or a control mixture of nonessential amino acids (Control) as indicated. Immune phenotypes were identified by indirect immunofluorescence assay and flow cytometry as described in Materials and Methods. Values are means ± SEM ($n \geq 6$/group).

* Indicates a significant effect of the tumour in rats fed the control diet ($p < 0.05$, by ANOVA). Means within a row that do not share a common superscript letter are significantly different ($p < 0.02$, by ANOVA and least squares means).

**TABLE 8.6 Immune Cell Phenotypes Expressing the Interleukin-2 Receptor (CD25) After 48 h Splenocyte Stimulation With Concanavalin A[1]**

| *Animal Treatment* | **Healthy** | **Tumour** | **Tumour** | **Tumour** | **Tumour** |
|---|---|---|---|---|---|
| *Diet* | **Control** | **Control** | **Gln** | **Arg** | **OKG** |
| **Immune Cell Phenotype** | % of total immune cells in spleen | | | | |
| $CD25^+$ $CD5^+$ T cells | 48 ± 5 | $51 \pm 3^a$ | $53 \pm 2^{ab}$ | $50 \pm 2^a$ | $56 \pm 2^b$ |
| $CD25^+$ $CD4^+$ T helper cells | 37 ± 3 | 37 ± 3 | 40 ± 2 | 36 ± 2 | 40 ± 2 |
| $CD25^+$ $CD8^+$ T suppressor/cytotoxic cells | 15 ± 2 | 18 ± 1 | 17 ± 1 | 18 ± 1 | 19 ± 1 |
| $CD25^+$ B cells | 32 ± 5 | 32 ± 3 | 34 ± 4 | 31 ± 4 | 30 ± 3 |
| $CD25^+$ Macrophages | 6.8 ± 0.9 | 7.4 ± 1.1 | 7.9 ± 1.2 | 10.5 ± 1.7 | 9.3 ± 1.4 |
| $CD25^+$ $CD28^+$ cells | 45 ± 4 | 48 ± 4 | 52 ± 3 | 50 ± 2 | 49 ± 2 |

[1] Splenocytes were isolated from healthy or tumour-bearing rats fed semi-purified diets supplemented with glutamine (Gln), arginine (Arg), ornithine α-ketoglutarate (OKG) or a control mixture of nonessential amino acids (Control) as indicated. Isolated splenocytes were stimulated for 48 h with Concanavalin A (5 mg/L) and immune phenotypes were identified by indirect immunofluorescence assay and flow cytometry as described in Materials and Methods. Values are means ± SEM ($n \geq 6$/group). Means within a row that do not share a common superscript letter are significantly different ($p < 0.05$, by ANOVA and least squares means).

TABLE 8.7. Splenocyte Cytokine Production After Mitogen Stimulation[1]

| *Animal Treatment*<br>*Diet* | **Healthy**<br>**Control** | **Tumor**<br>**Control** | **Tumor**<br>**Gln** | **Tumor**<br>**Arg** | **Tumor**<br>**OKG** |
|---|---|---|---|---|---|
| **Concanavalin A** | | | pg/ml (3 x $10^6$ cells/ml) | | |
| IL-2 | 4571 ± 437 | 4416 ± 436 | 4388 ± 182 | 4839 ± 352 | 4731 ± 371 |
| IFN-γ | 3601 ± 304 | 3343 ± 577 | 3725 ± 640 | 3728 ± 487 | 3260 ± 361 |
| TNF-α | 525 ± 48 | 568 ± 77 | 595 ± 53 | 590 ± 76 | 504 ± 48 |
| **PMA + Ionomycin** | | | | | |
| IL-2 | 13343 ± 1314 | 10692 ± 1571 | 11804 ± 1329 | 13033 ± 1651 | 13600 ± 1623 |
| IFN-γ | 2799 ± 251 | 2214 ± 449 | 2608 ± 490 | 3529 ± 502 | 2349 ± 366 |
| TNF-α | 728 ± 72 | 622 ± 98 | 716 ± 100 | 775 ± 75 | 735 ± 68 |

[1] Splenocytes were isolated from healthy or tumour-bearing rats fed semi-purified diets supplemented with glutamine (Gln), arginine (Arg), ornithine α-ketoglutarate (OKG) or a control mixture of nonessential amino acids (Control) as indicated. Isolated splenocytes were stimulated for 48 h with Concanavalin A (5 mg/L) or Phorbol Myristate Acetate (PMA, 30 μg/L) plus Ionomycin (0.75 μmol/L) and cytokine production was determined by ELISA as described in Materials and Methods. Values are means ± SEM for splenocytes stimulated with Concanavalin A or PMA plus Ionomycin for 48 h ($n \geq 6$/group). No significant differences were found.

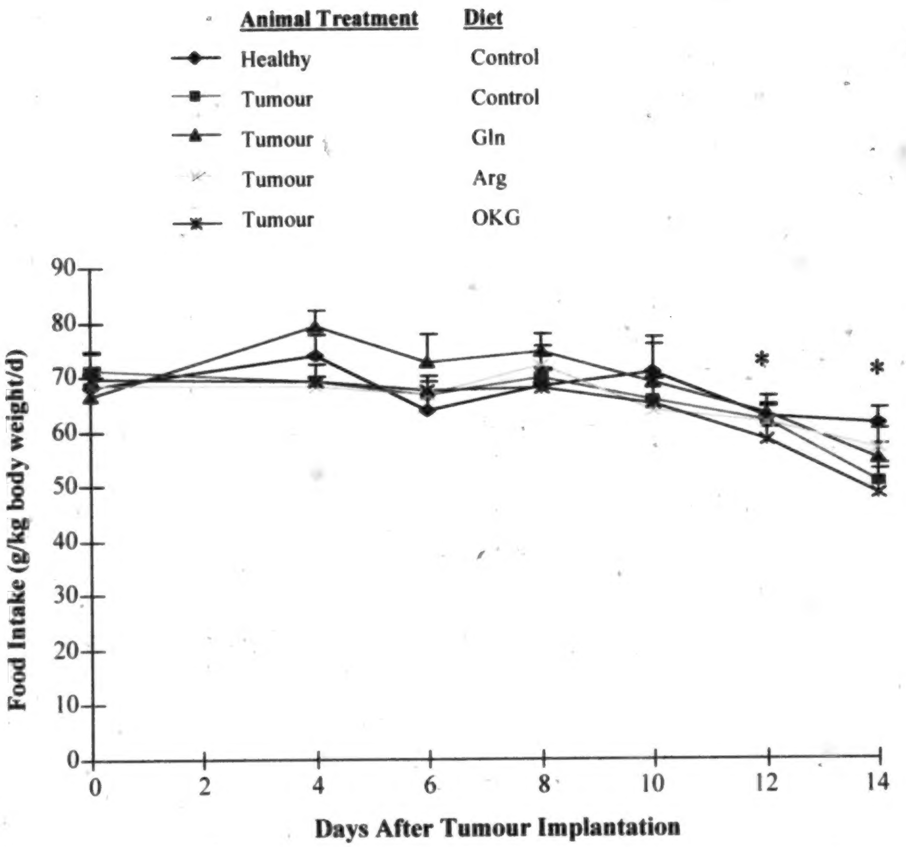

**FIGURE 8.1 Food Intake of Healthy and Tumour-Bearing Rats Fed Different Diets.** Food was weighed every 2 d and calculated as gram of food per kg rat body weight per day (g/kg body weight/d). Values are means ± SEM ($n \geq 7$/group). Neither tumour nor diet significantly affected food intake as determined by repeated measures ANOVA. Food intake decreased starting at day 12 (* $p < 0.05$, by repeated measures ANOVA).

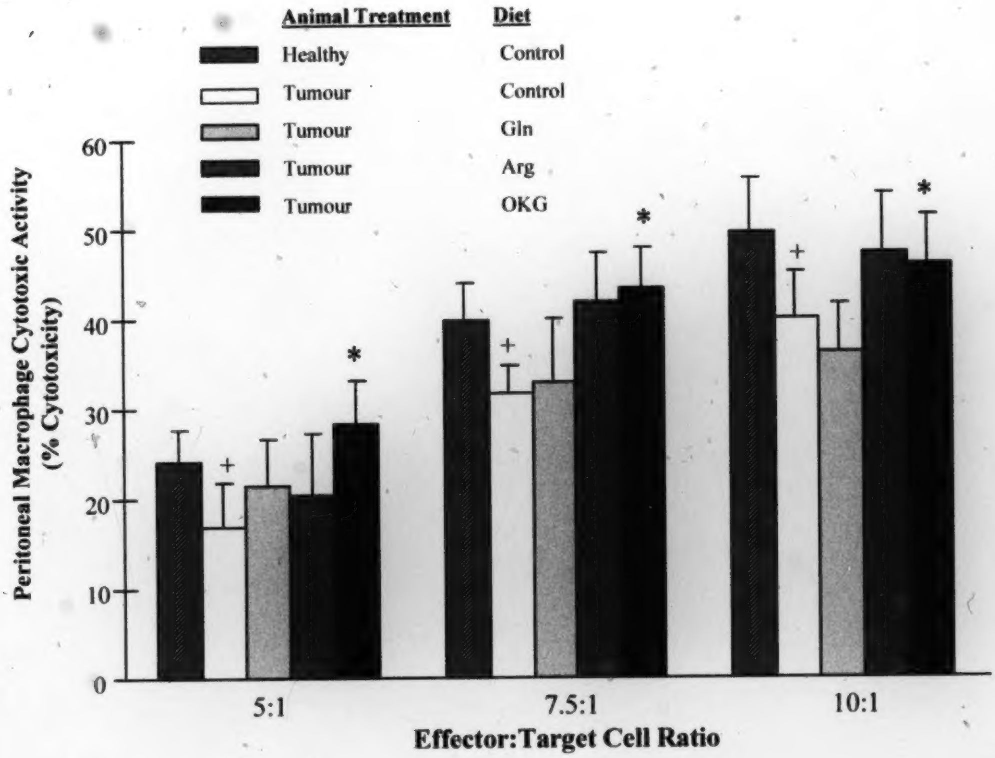

**FIGURE 8.2 Macrophage Cytostatic Activity Against P815 Cells at Different Effector:Target Cell Ratios.** Peritoneal macrophages were cocultured with P815 target cells in the presence of [methyl-$^3$H]-thymidine as described in Materials and Methods. After 6 h, cells were harvested and the radioactivity incorporated into the target cells was determined. Macrophage cytostatic activity was determined using the following formula: Cytostatic activity = 100 – (100 x cpm TM/cpm T) where cpm TM = radioactivity (counts per minute) in target cell: macrophage coculture and cpm T = radioactivity in target cell culture. Bars represent means ± SEM ($n \geq 7$/group). A significant effect of the tumour in rats fed the control amino acid diet was determined by one-way split-plot (repeated measures) ANOVA († $p < 0.05$). Similarly, a significant effect of diet in tumour-bearing rats was determined by one-way split-plot (repeated measures) ANOVA (* $p < 0.05$).

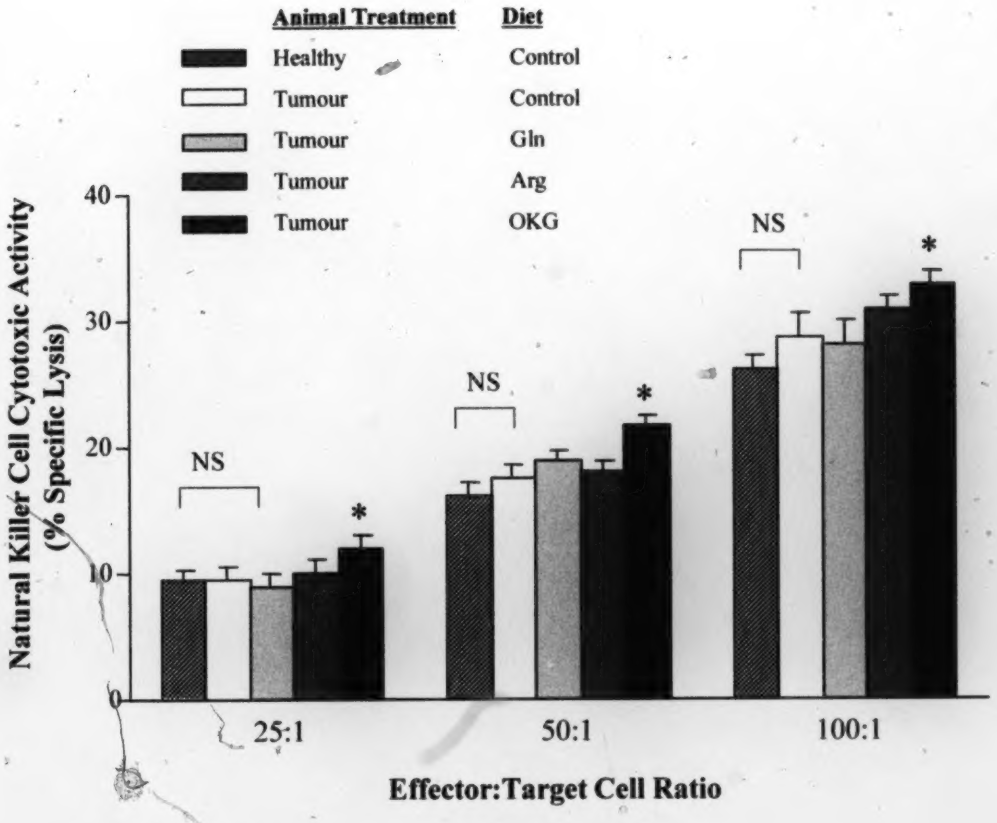

**FIGURE 8.3 Splenocyte Natural Killer Cell Cytotoxicity Against YAC-1 Cells at Different Effector:Target Cell Ratios.** Natural killer (NK) cell cytotoxic activity is expressed as the percentage of specific lysis, which is equal to 100 x (mean experimental $^{51}$Cr release from labeled YAC-1 cells – mean spontaneous $^{51}$Cr release)/(mean maximum $^{51}$Cr release – mean spontaneous $^{51}$Cr release). Bars represent means ± SEM ($n \geq 10$/group). The tumour did not significantly affect NK cell cytotoxic activity (NS, $p > 0.05$ by one-way split-plot (repeated measures) ANOVA). However, there was a significant effect of diet in tumour-bearing rats (* $p < 0.01$, by one-way split-plot (repeated measures ANOVA).

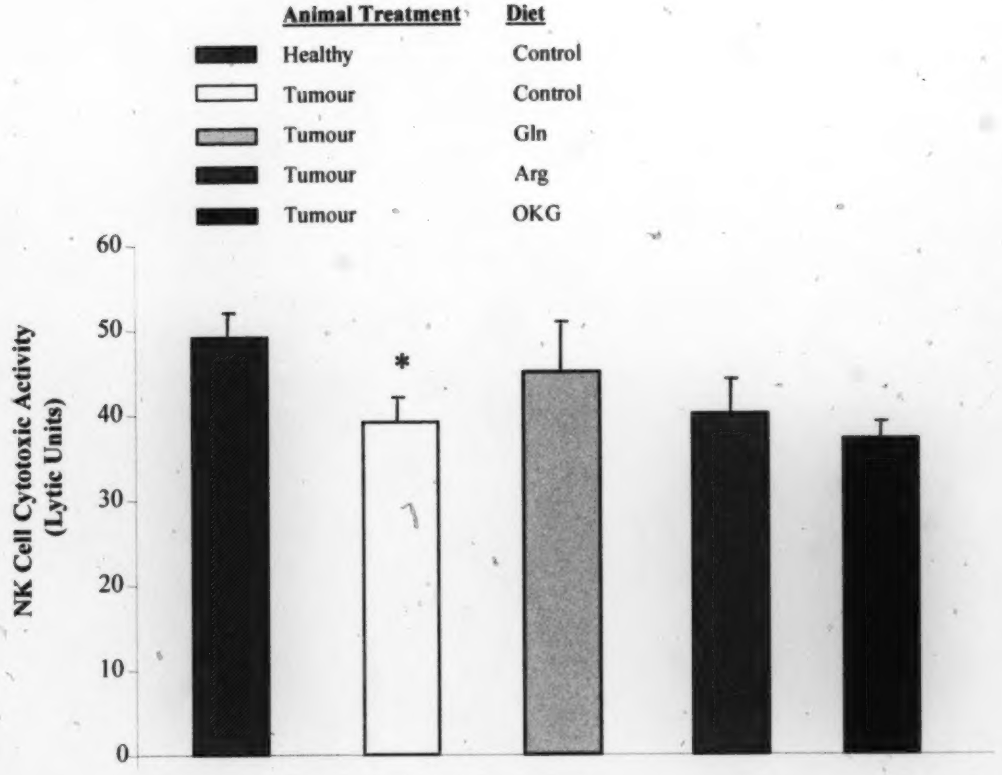

**FIGURE 8.4 Splenocyte Lytic Activity.** Lytic units are defined as the number (x $10^{-3}$) of natural killer (NK) cells required to cause 20% lysis of target cells. Bars represent means ± SEM ($n \geq 6$/group). For rats fed the control diet, the effect of the tumour was analyzed by one-way ANOVA (* $p > 0.05$). For tumour-bearing rats, lytic units were not significantly different among diet groups (by one-way ANOVA).

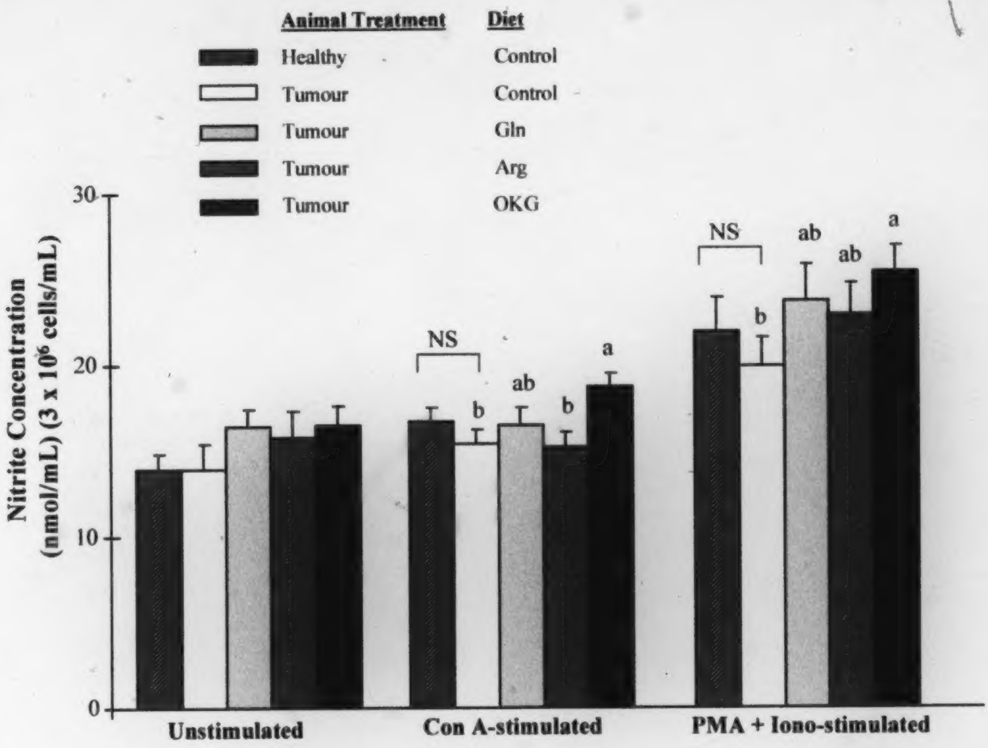

**FIGURE 8.5 Nitric Oxide Production by Unstimulated and Mitogen-Stimulated Splenocytes.** Cells were cultured without mitogen (unstimulated) or with either Concanavalin A (Con A, 5 mg/L) or Phorbol Myristate Acetate (PMA, 30 µg/L) plus Ionomycin (Iono, 0.75 µmol/L) for 48 h. The concentration of nitrite ($NO_2^-$) in splenocyte culture supernatants was determined by the Griess reaction as described in Materials and Methods. Bars represent means ± SEM ($n \geq 7$/group). For rats within the same treatment group, a paired *t* test was used to determine increased $NO_2^-$ production by stimulated splenocytes compared with unstimulated cells (statistics not shown). For rats fed the control diet, the effect of the tumour was analyzed by one-way ANOVA at each culture condition (NS, $p > 0.05$). For tumour-bearing rats, the effect of diet was analyzed by one-way ANOVA at each culture condition and least squares means were used to determine differences among groups. For each culture condition, means that do not share a common letter are significantly different ($p < 0.05$).

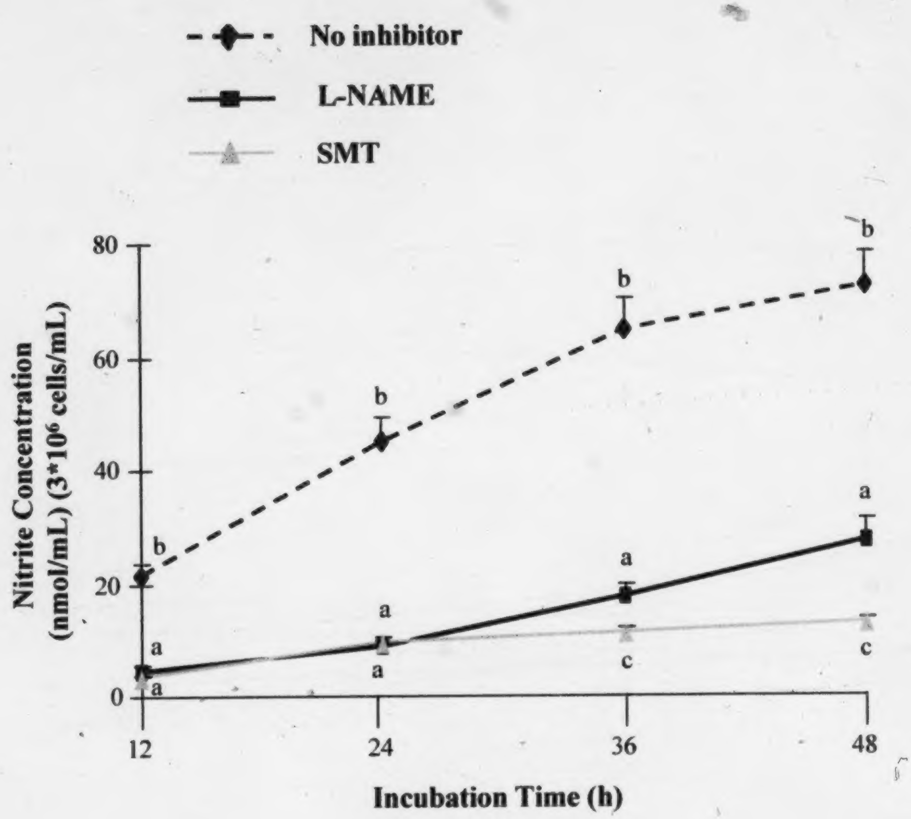

**FIGURE 8.6 Splenocyte Nitric Oxide Production and Nitric Oxide Synthase Inhibition.** Splenocytes were incubated without nitric oxide synthase inhibitor (maximal response, *n* = 69) or with either L-$N^G$-nitro-L-arginine methylester (L-NAME, 1200 nmol/$10^6$ cells, *n* = 69) or S-methylisothiourea (SMT, 120 nmol/$10^6$ cells, *n* = 36) for 12, 24, 36 or 48 h. The concentration of nitrite ($NO_2^-$) in splenocyte culture supernatants was determined by the Griess reaction as described in Materials and Methods. Bars represent means ± SEM. The inhibition was analyzed by a paired *t* test at each incubation time. Values that do not share a common letter are significantly different ($p < 0.05$).

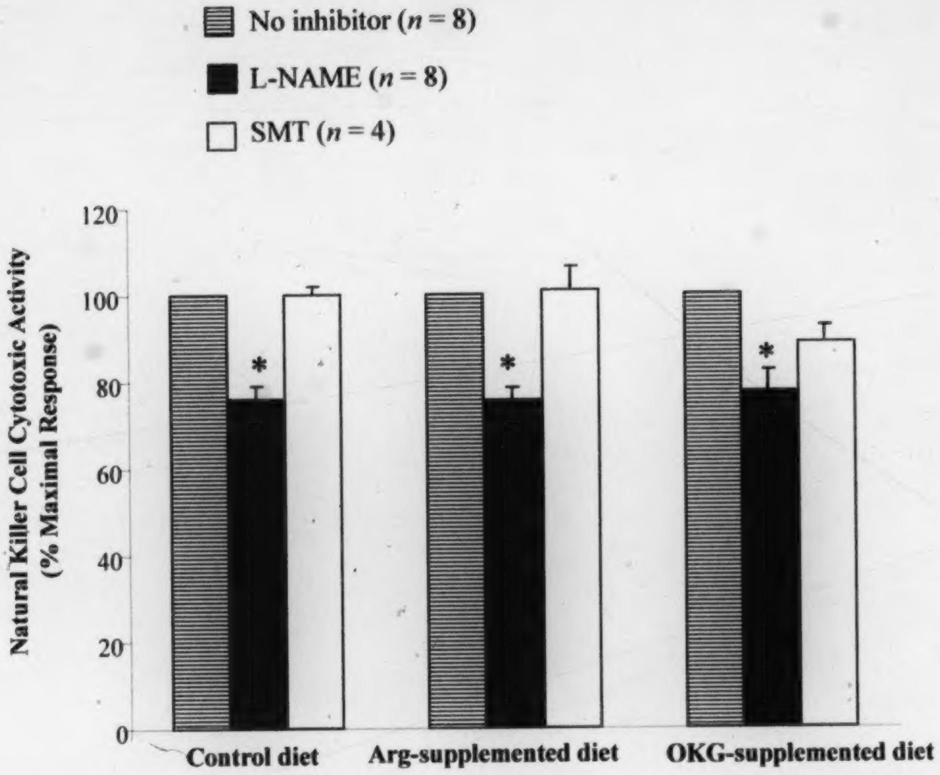

**FIGURE 8.7 Effect of Nitric Oxide Synthase Inhibitors on Natural Killer Cell Cytotoxic Activity in Tumour-Bearing Rats.** Splenocytes were incubated for 10 min at 37 °C without nitric oxide synthase inhibitor (maximal response) or with either L-$N^G$-nitro-L-arginine methylester (L-NAME, 1200 nmol/$10^6$ cells) or S-methylisothiourea (SMT, 120 nmol/$10^6$ cells). Splenocytes were then cultured with $^{51}$C-labelled YAC-1 cells for 4 h to achieve effector:target cell ratios between 25:1 and 100:1 as described in Materials and Methods. Natural killer (NK) cell cytotoxic activity is expressed as percentage of maximal response at the 100:1 ratio. Bars represent means ± SEM. For each diet group, the effect of nitric oxide synthase inhibitor on NK cell cytotoxic activity was determined by one-way ANOVA (*$p < 0.05$ compared with maximal response).

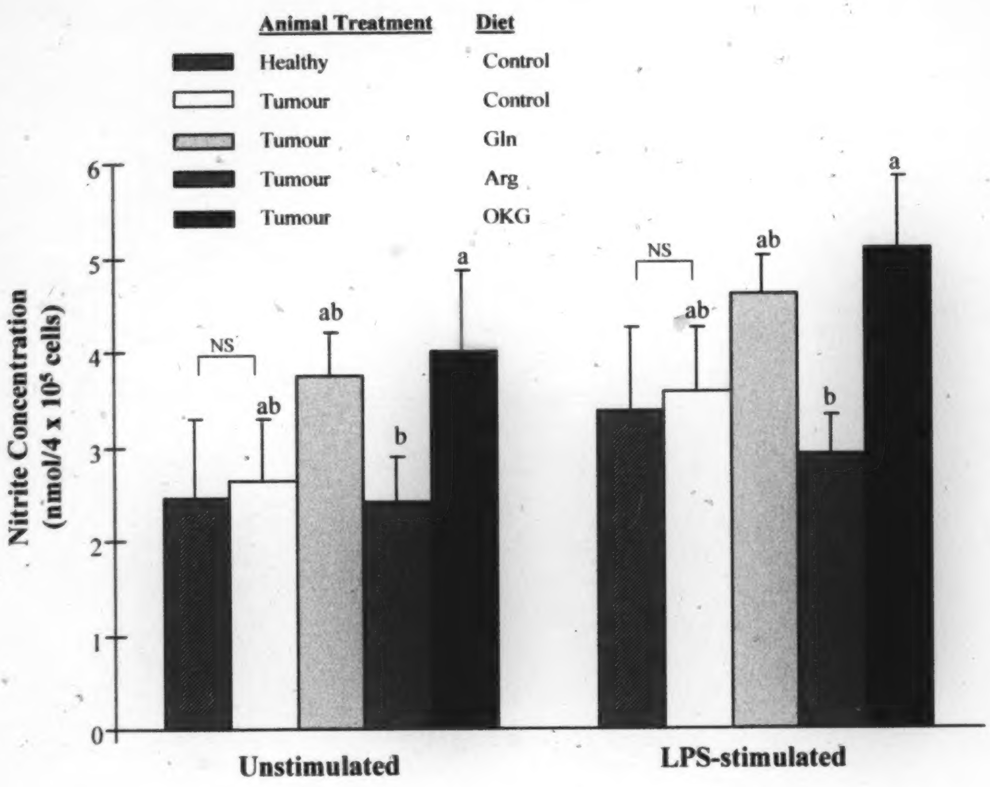

**FIGURE 8.8 Nitric Oxide Production by Unstimulated and Lipopolysaccharide Stimulated Peritoneal Macrophages.** Peritoneal macrophages were incubated without mitogen (unstimulated) or with Lipopolysaccharide (LPS) for 24 h. The concentration of nitrite ($NO_2^-$) in macrophage culture supernatants was determined by the Griess reaction as described in Materials and Methods. Bars represent means ± SEM ($n \geq 7$/group). For rats fed the control diet, the effect of the tumour was analyzed by one-way ANOVA at each culture condition (NS, $p < 0.05$). For tumour-bearing rats, the effect of diet was analyzed by one-way ANOVA at each culture condition. For each culture condition, means that do not share a common letter are significantly different ($p < 0.05$) as identified by least squares means.

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# 9. GENERAL SUMMARY AND DISCUSSION

## 9.1 SUMMARY OF RESULTS

The objectives of this research were to test the following general working hypotheses:

### I. DIETARY FAT COMPOSITION ALTERS THE FATTY ACID COMPOSITION OF PRE-ACTIVATED AND ACTIVATED IMMUNE CELLS

This hypothesis was supported by the results reported in Chapters 3, 4 and 6. Specifically, it was hypothesized that:

**A. Dietary long-chain n-3 fatty acids will increase n-3 fatty acid incorporation into immune cell membrane phospholipids.**

The results reported in Chapters 3, 4 and 6 supported this hypothesis. Freshly isolated splenocytes from rats fed dietary fish oil-derived long-chain n-3 fatty acids had a significantly higher n-3 fatty acid content and a significantly lower n-6/n-3 ratio in the major immune cell phospholipids phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI).

**B. The fatty acid composition of immune cell membrane phospholipids will be altered with mitogen activation. The diet-induced increase in n-3 fatty acid incorporation in immune cell phospholipids will be maintained in an activated state.**

These hypotheses were supported by the results reported in Chapters 4 and 6. Cell culture (without mitogen) and mitogen activation significantly altered the fatty acid composition of immune cell phospholipids isolated from rats fed different diets (Chapter 4). In general, the polyunsaturated fatty acids were most affected (Chapter 4). Overall, many of the diet-induced changes, including the increase in n-3 fatty acids, in immune cell phospholipids were maintained during 48 h culture in the absence or presence of mitogen, although results varied with the presence of a tumour, dietary composition, the activation state of immune cells, and the individual phospholipid fraction (Chapter 4 and 6). Generally, diet-induced changes in levels of n-6 and n-3 fatty acids were better maintained after activation in healthy rats compared with tumour-bearing rats (Chapter 6).

### C. Exercise-training will alter the fatty acid composition of immune cell membrane phospholipids.

Our findings in Chapter 3 supported this hypothesis, although the effects of exercise-training on immune cell membrane composition were limited. Furthermore, there was a significant interaction between dietary long-chain n-3 fatty acids and exercise-training on the fatty acid composition of immune cells. For example, in low n-3-fed rats, exercise-training decreased the n-6/n-3 ratio in PC, while in high n-3 fed rats exercise-training increased total n-3 fatty acids in PE (Chapter 3).

### D. The incorporation of n-3 fatty acids into mammary tumour cell membrane phospholipids will be enhanced when long-chain n-3 fatty acids are fed in a low P/S, compared with a high P/S, diet.

The experiments described in Chapter 5 provided evidence for this hypothesis. Tumour cells from rats fed long-chain n-3 fatty acids had a higher n-3 fatty acid content in the major membrane phospholipids PC, PE and PI and the magnitude of increase in n-3 fatty acid incorporation was greater when fed in a low polyunsaturated/saturated fat (P/S) diet.

## II. THE TUMOUR-BEARING STATE ALTERS THE FATTY ACID COMPOSITION OF PRE-ACTIVATED AND ACTIVATED IMMUNE CELLS AND AFFECTS HOST IMMUNE FUNCTION.

This hypothesis was supported by the results reported in Chapters 5, 6 and 8. Specifically, it was hypothesized that:

**E. The fatty acid composition of immune cell membrane phospholipids in the pre-activated and activated state will be altered in rats implanted with a mammary tumour compared with healthy rats.**

This hypothesis was supported by our results described in Chapter 6. The tumour significantly altered the fatty acid composition of phospholipids in freshly isolated and mitogen-activated immune cells, but some of the tumour-induced effects differed with dietary fat composition. For example, the tumour significantly altered n-3 and n-6 fatty acid levels in freshly isolated splenocytes and mitogen-activated cells, but only in the low n-3 diet group.

**F. Host immune function will be suppressed in rats implanted with a tumour compared with healthy rats.**

We verified this hypothesis for certain immune parameters. In Chapters 5 and 6, the proportion of activated ($CD25^+$) $CD4^+$ T cells, macrophages, $CD28^+$ cells and B cells after mitogen (PMA plus Ionomycin) stimulation, proliferative response to mitogen and natural killer (NK) cell cytotoxicity were significantly suppressed in rats bearing a mammary tumour, although some of the tumour-induced effects varied with dietary fat composition. For example, tumour burden had a suppressive effect on NK cell cytotoxicity, but only when rats were fed the low n-3 diet without long-chain n-3 fatty acids (Chapter 5). In Chapter 8, the major changes in the tumour-bearing state were a decreased proportion of macrophages in spleen and suppressed macrophage cytostatic activity. However, hypothesis F was not true in general since other immune parameters assayed were enhanced in tumour-bearing rats. For example, CD25 expression on T cells after Con A stimulation, type-1 cytokine (IFN-γ and TNF-α) production by mitogen-stimulated splenocytes, and nitric oxide production were significantly increased in tumour-bearing, compared with healthy, rats (Chapters 5 and 6).

## III. DIETARY LONG-CHAIN N-3 FATTY ACIDS INHIBIT TUMOUR GROWTH THROUGH AN UPREGULATION OF MEMBRANE-DEPENDENT IMMUNE DEFENSES, INCLUDING IMMUNE CELL ACTIVATION.

Specifically, it was hypothesized that:

G. **Dietary long-chain n-3 fatty acids fed at a physiological level which could potentially be achieved in the human diet will enhance anticancer immune defense in healthy rats, including cell activation NK cell cytotoxicity, and production of cytokines and nitric oxide.**

This hypothesis was supported by the results presented in Chapters 3, 4, 5 and 6. Dietary long-chain n-3 fatty acids enhanced expression of immune cell activation markers (transferrin receptor, CD71) and NK cell cytotoxicity (Chapters 3, 4 and 5), nitric oxide production (Chapter 4 and 5) and type-1 cytokine production (Chapter 5 and 6) in healthy rats.

H. **Exercise-training of low-intensity will enhance anticancer immune defense, including immune cell activation and NK cell cytotoxicity.**

Evidence for this hypothesis was reported in Chapter 3. Specifically, in rats fed the low n-3 diet, low-intensity exercise-training increased the proportion of T and B cells that were activated ($CD71^+$) after mitogen stimulation, an observation indicative of immune enhancement.

I. **The combination of dietary long-chain n-3 fatty acids and low-intensity exercise-training will further enhance anticancer immune defense (i.e. they will have a synergistic benefit on immune defense).**

This hypothesis was not supported by our results. The combination of dietary long-chain n-3 fatty acids and low-intensity exercise-training did not have a synergistic effect on immune cell activation and NK cell cytotoxicity in healthy rats (Chapter 3).

We investigated the following four hypotheses regarding the role of dietary long-chain n-3 fatty acids in tumour growth inhibition and upregulation of various anticancer immune defenses:

J. **Dietary long-chain n-3 fatty acids will inhibit mammary tumour growth when fed at a physiological level which could potentially be achieved in the human diet.**

K. **The efficacy of dietary long-chain n-3 fatty acids to reduce tumour growth will differ with the P/S ratio of the diet. Specifically, long-chain n-3 fatty acids will be more effective at inhibiting tumour growth when fed in a low P/S, compared with a high P/S, diet.**

L. **Dietary long-chain n-3 fatty acids exert their cancer inhibitory effect through upregulation of the host immune system, including enhanced cell activation, natural killer cell cytotoxicity, and production of cytokines and nitric oxide.**

M. **The efficacy of dietary long-chain n-3 fatty acids to improve immunity will differ with the P/S ratio of the diet. Specifically, the upregulation of anticancer immune defense by dietary long-chain n-3 fatty acids will be enhanced when fed in a low P/S, compared with a high P/S, diet.**

Dietary long-chain n-3 fatty acids did not significantly inhibit tumour growth (Chapters 5 and 6, hypothesis J not supported). However, long-chain n-3 fatty acids were more effective at reducing tumour growth when fed in a low P/S diet, but this effect did not reach statistical significance (Chapter 5, hypothesis K not supported). Despite no significant effect of diet on tumour growth, membrane-dependent immune defenses, including immune cell activation ($CD25^+$ $CD8^+$ T cells), NK cell cytotoxicity, type-1 cytokine (IFN-$\gamma$ and TNF-$\alpha$) and nitric oxide production were upregulated by dietary long-chain n-3 fatty acids fed in a low P/S diet (Chapters 5 and 6, hypothesis L partially supported). Furthermore, in tumour-bearing rats, the upregulation of immune defense by dietary long-chain n-3 fatty acids was enhanced when fed in a low P/S diet (Chapter 5, hypothesis M supported).

## IV. DIETARY LONG-CHAIN N-3 FATTY ACIDS INHIBIT TUMOUR GROWTH THROUGH A REDUCTION IN CELLULAR PROLIFERATION, AN INCREASE IN APOPTOSIS, AND AN ALTERATION IN EXPRESSION OF GROWTH-RELATED PROTEINS.

The inhibition of tumour growth by the long-chain n-3 fatty acid DHA (C22:6n-3) and potential mechanisms of this inhibitory effect were reported in Chapter 7. Specifically, it was hypothesized that:

**N. The addition of fatty acids to the tumour cell culture media will modify the fatty acid composition of tumour cell membrane phospholipids.**

**O. Incubation of breast cancer cells with the long-chain n-3 fatty acid, DHA, will reduce cellular proliferation relative to control, but will not be cytotoxic to the cancer cells.**

**P. The inhibitory effect of DHA on tumour cell proliferation is not due to a deficiency of LA in the cell culture media. Specifically, the addition of DHA plus LA to the cell culture media will reduce tumour cell proliferation relative to control.**

**Q. The inhibition of tumour cell proliferation by DHA will be related to cell cycle arrest and an increase of tumour cells in $G_0$ (quiescent phase).**

**R. The addition of DHA to the cell culture media will decrease the expression of various cell cycle regulatory proteins, such as the D-type cyclins and cyclin B1.**

**S. The inhibition of tumour cell proliferation by DHA will be related to decreased expression of proliferation-related proteins, such as proliferating**

**cell nuclear antigen (PCNA) and proliferation-related kinase (PRK).**

T. **The inhibition of tumour cell proliferation by DHA will be related to increased expression of tumour-suppressor proteins, such as p53 and retinoblastoma protein (pRb).**

U. **Incubation of breast cancer cells with DHA will increase apoptosis relative to control.**

Incubation with DHA increased the n-3 fatty acid composition of MDA-MB-231 breast cancer cell phospholipids, with a concomitant decrease in n-6 fatty acids (hypothesis N supported), and reduced cell proliferation relative to LA (C18:2n-6), without inducing a cytotoxic effect (hypothesis O supported). Furthermore, the growth inhibitory effect of DHA was not due to a deficiency of LA in the media since cells incubated with LA + DHA also had lower proliferation compared with LA alone (hypothesis P supported). The mechanism for the DHA-induced growth inhibition was not due to an alteration in the distribution of breast cancer cells in specific cell cycle phases (hypothesis Q not supported), expression of D-type cyclins (hypothesis R not supported), or proliferation-related proteins (PCNA and PRK) in breast cancer cells (hypothesis S not supported). However, DHA significantly decreased expression of cyclin B1 in S phase of the cell cycle (hypothesis R partially supported) and increased levels of p53 and pRb tumour suppressor proteins in breast cancer cells (hypothesis T supported). Finally, DHA did not increase breast cancer cell apoptosis in our model (hypothesis U not supported).

## V. DIETARY GLN, ARG, AND OKG UPREGULATE ANTICANCER IMMUNE FUNCTION IN THE TUMOUR-BEARING STATE.

The effects of dietary supplementation with glutamine (Gln), arginine (Arg), and ornithine α-ketoglutarate (OKG) on anticancer immune defense in tumour-bearing rats were reported in Chapter 8. Specifically, it was hypothesized that:

**V. Dietary supplementation with Gln, Arg, and OKG will reduce tumour growth compared with a diet containing nonessential amino acids.**

This hypothesis was not supported as there was no effect of these dietary amino acids on tumour growth in rats. (Chapter 8).

**W. Dietary supplementation with Gln, Arg, and OKG will upregulate anticancer immune defenses, such as immune cell activation, the cytotoxic activity of marcophages and NK cells, and the production of cytokines and nitric oxide.**

This hypothesis was partially supported by the results reported in Chapter 8. There were limited or no significant effects of Gln and Arg on the anticancer immune parameters assayed. However, OKG upregulated NK cell cytotoxicity and macrophage cytostatic activity, the proportion of activated T cells, and nitric oxide production after mitogen stimulation.

## 9.2 GENERAL DISCUSSION

### Diet and Tumour Growth

The central issue we addressed was the anticancer effect of dietary long-chain n-3 fatty acids. Previous studies have claimed to demonstrate such effects in animal models (Ip 1997). However, we showed that dietary fish oil-derived long-chain n-3 fatty acids fed in either a high P/S or a low P/S diet do not significantly inhibit mammary tumour growth in a rat model. One reason long-chain n-3 fatty acids did not inhibit tumour growth *in vivo* may have been our dietary design. Many animal model studies that have reported growth inhibitory effects of dietary long-chain n-3 fatty acids have fed diets with very high levels of fish oil (e.g. 100% of fat in the diet from fish oil is a common diet used in experimental studies). Clearly, it is questionable whether such an extreme level of fish oil has any implications for the design of human dietary intervention trials, when it would be extremely difficult to make such a substantial change in the habitual dietary intake of individuals in the population. In addition, most animal model studies to date have focused on single dietary oils or mixtures of two oils, which often resulted in levels of long-chain n-3 fatty acids far above those consumed by most humans as well as very low intakes of the essential n-6 fatty acid LA. This is a critical consideration in diet and cancer studies since LA has been shown to be essential for tumour growth (Ip et al. 1985). Furthermore, since human dietary fat intake is usually a mixture of lipids from various sources, many studies have failed to use diets that are representative of their normal consumption patterns. Finally, it has been suggested that the ratio of n-6 to n-3 fatty acids in the diet, not the absolute levels of fatty acids, is critical for the anticancer effect of long-chain n-3 fatty acids. Previous studies have reported that n-6/n-3 fatty acid ratios from 2 to 0.5 are optimal for anticancer benefits (Calviello et al. 1998, Cohen et al. 1993, Abou-el-Ela et al. 1989, Karmali et al. 1984). However, these ratios are considerably lower than that reported in a recent study where Alberta residents were found to consume an average n-6/n-3 fatty acid ratio of 7 (Alberta Nutrition Survey, unpublished results). In short, the physiological relevance of many reported anticancer effects by long-chain n-3 fatty acids is uncertain due to dietary design.

Our experimental diets contained ~ 40% of energy from fat from a mixture of sources [safflower oil, hard beef tallow, linseed oil (low n-3 diet only) or fish oil (high n-3 diet only)] and were designed to reflect the level and composition of fat in the North American diet. In comparison with many previously reported studies, we fed long-chain n-3 polyunsaturated fatty acids at a more physiological level with respect to the human diet (0 versus 5 g/100 g of total fat in the low and high n-3 diets, respectively). In terms of P/S ratio, our diets were designed to reflect either that currently recommended by several health agencies (P/S = 1) or to represent the P/S ratio consumed by a large segment of the North American population (P/S = 0.35). The n-6/n-3 fatty acid ratios in the long-chain n-3 fatty acid-supplemented diets were 7.8 and 3.8 in the high and low P/S diets, respectively. Overall, our more physiologically relevant diets contained lower absolute levels of long-chain n-3 fatty acids (and a considerably higher corresponding n-6/n-3 ratio) than many previous studies animal studies which have shown a tumour inhibitory effect of dietary long-chain n-3 fatty acids (Calviello et al 1998, Cohen et al 1993, Abou-el-Ela et al 1989, Karmali et al 1984) and this may explain why we did not see a significant effect on tumour growth.

Our finding that dietary long-chain n-3 fatty acids did not inhibit mammary tumour growth may also be related to the nature of rodent transplantable tumour models.

We chose the R3230AC mammary adenocarcinoma, a transplantable rat tumour that shares many similarities with the human disease (Hillyard et al. 1980) and whose growth *in vivo* has been shown to be reduced by dietary fish oil-derived n-3 fatty acids (Karmali et al 1984). Although experimental tumours are not identical to spontaneously occurring human tumours, they can provide valuable models to examine therapeutic principles, such as the impact of potential anticancer nutrients. However, a general problem with this sort of methodology is the short time frame inherent in transplantable tumour model studies. The rapid progression of the tumour may not have allowed enough time for effects of dietary long-chain n-3 fatty acids to manifest themselves. However, the length of time that we fed our diets (17 days post-implantation) was sufficient to change tumour cell membrane composition (Chapter 5) and also other studies have demonstrated R3230AC growth inhibition by long-chain n-3 fatty acids at 21 days post-implantation (Karmali et al 1984). These facts suggest that this feature of transplantable models is not responsible for the lack of a diet-induced growth effect in our study. We did not continue our study over a longer time period after tumour implantation because we wanted to avoid the onset of complicating factors, such as malnutrition and cachexia which could have influenced both tumour growth and immune function.

Although dietary long-chain n-3 fatty acids did not significantly inhibit mammary tumour growth *in vivo*, our results do suggest a trend towards reduced tumour growth when long-chain n-3 fatty acids were fed in a low P/S diet as opposed to a high P/S diet (Chapter 5). Overall, this trend, combined with our finding that the long-chain n-3 fatty acid DHA significantly reduced proliferation of MDA-MB-231 human breast cancer cells in our *in vitro* model (Chapter 7), support findings in the literature and provide motivation for our continued study of the role of dietary long-chain n-3 polyunsaturated fatty acids in breast cancer. It remains possible that the R3230AC mammary tumour might respond to dietary long-chain n-3 fatty acids in other experimental situations, especially given that many of the host antitumour immune parameters enhanced by dietary long-chain n-3 fatty acids (discussed below) are important for prevention of cancer recurrence. In future, the R3230AC mammary tumour model could be used to study the role of long-chain n-3 fatty acids in a situation that more closely approximates the human situation, such as secondary disease prevention following surgical removal of the tumour, possibly in addition to chemotherapy or other cancer treatments.

Despite the fact that our cancer model provided us with information about the role of long-chain n-3 fatty acids in tumour growth, the extension of such results to the human situation must be done with caution. Clearly, tumour models reflecting all types of human cancers (of which there are more than 100) have not been tested. This makes the interpretation of existing literature and the application of animal model data to the human disease extremely difficult. This issue was raised in Chapter 8 where we studied three additional dietary nutrients for their ability to decrease tumour growth in rats. Our results demonstrated that neither glutamine (Gln), arginine (Arg), nor ornithine α-ketoglutarate (OKG) significantly affected growth of the Morris hepatoma 7777, another transplantable rodent tumour model. Although our results are consistent with many previous studies in this field, the literature in this area is difficult to interpret, arising from the fact that there are currently at least 30 published papers focusing on Gln, Arg and OKG supplementation and tumour growth and these have used a wide variety of tumour types and hosts, tumour immunogenicity, both enteral and parenteral feeding regimens, levels of amino acid supplementation ranging from 1 to 10% w/w of the diet, tumour burdens ranging from < 1 to 30% of host body weight and a variety of "control diets". Not surprisingly, these studies have generated very diverse results and highlight the complexity of understanding the role of amino acids in cancer. Clearly, the application of animal model data to the human situation in this case is not straightforward and requires further study.

### Diet, Exercise and Immunity in a Healthy Rat Model

The mechanism for the putative anticancer benefits of dietary long-chain n-3 fatty acids is not known. We therefore investigated whether the tumour suppressor effect of long chain n-3 fatty acids may occur, in part, via alterations in the host immune system. We found that dietary long-chain n-3 fatty acids enhanced various anticancer immune responses in both healthy and tumour-bearing rats. In general, the current literature has concluded that long-chain n-3 polyunsaturated fatty acids are immunosuppressive. However, our findings indicate the opposite in a healthy rat model. Again, we think our findings are more physiologically relevant since we fed lower levels of long-chain n-3 fatty acids in either a high P/S or low P/S diet as discussed above. As well, many studies that have reported immunosuppressive effects of long-chain n-3 fatty acids, have used models other than the healthy state, such as autoimmune diseases (rheumatoid arthritis, systemic lupus) and various infection models (Calder 1998, Harbige 1998). Admittedly, the ability of n-3 fatty acids to suppress macrophage function has been consistently demonstrated and we did not assess this immune parameter in our fatty acid studies. However, we observed that dietary long-chain n-3 fatty acids significantly enhanced various immune parameters in healthy rats, including the proportion of activated ($CD71^+$) immune cells after mitogen stimulation, NK cell cytotoxicity, and splenocyte nitric oxide and TNF-α production (Chapters 3-6). In Chapter 4, immune cell proliferative response to Con A (primarily a T cell mitogen) was suppressed in healthy rats fed a high P/S diet supplemented with long-chain n-3 fatty acids. We do not know if this suppression in proliferative response to mitogen is physiologically relevant in a healthy state and more work is needed to compare this *in vitro* suppression with the *in vivo* situation in a healthy model. Moreover, long-chain n-3 fatty acids fed in a low P/S diet, which better represents current intake patterns in humans, did not affect proliferation of immune cells isolated from healthy rats (Chapter 6). Our findings are important because feeding a physiological level of long-chain n-3 fatty acids (either in a high P/S or a low P/S diet) enhanced immune function in healthy rats, such as NK cell cytotoxicity and nitric oxide production, which are relevant to anticancer defense. The current conception that long-chain n-3 fatty acids are generally immunosuppressive has been difficult to overcome and has been the basis of opposition to the addition of the long-chain n-3 fatty acid DHA to infant formula. Our work provides evidence to support clinical studies of feeding long-chain n-3 fatty acids to enhance immune function in healthy individuals. Although upregulated immune function was not found to impact directly on tumour growth in our model, we suggest that enhanced immunity may have other health benefits, such as cancer prevention over the longer term, which require further study in a healthy model. Future work should also determine the distinct roles of EPA and DHA, the two main long-chain n-3 fatty acids in fish oil, in anticancer immune defense.

Our results also demonstrated that low-intensity exercise-training upregulated immune function in healthy rats (Chapter 3). However, the more important finding with respect to dietary long chain n-3 fatty acids was that, although long chain n-3 fatty acids and exercise-training were immunostimulatory on an individual basis, the combination of dietary long-chain n-3 fatty acids and low to moderate-intensity exercise-training did not further enhance the immune parameters assayed (immune cell activation and NK cell cytotoxicity) Thus, our results suggest that the immune responses produced by feeding long-chain n-3 fatty acids and exercise-training are not synergistic. These findings may help to explain current controversies in exercise immunology literature, since diet is infrequently controlled in exercise studies. To our knowledge, this was the first report of the interaction between dietary n-3 fatty acids and exercise-training with respect to immunocompetence. Furthermore, since there is an increasing amount of evidence implicating both dietary long chain n-3 fatty acids and regular, low-intensity exercise as playing beneficial roles in cancer prevention, our findings may be important when developing clinical strategies for cancer treatment.

### Diet and Immunity in a Tumour-Bearing Rat Model

When we studied the role of dietary long-chain n-3 fatty acids in altering immune responses in tumour-bearing rats as opposed to healthy rats, we obtained different results. In particular, long-chain n-3 fatty acids fed in the diet at physiological levels enhanced host immune parameters involved in antitumour defense when fed in a low P/S diet, but not a high P/S diet (Chapter 5). Specifically, tumour-bearing rats fed long-chain n-3 fatty acids in a low P/S diet had enhanced NK cell cytotoxicity, activated $CD8^+$ T cells and type-1 cytokine (IFN-$\gamma$ and TNF-$\alpha$) production after mitogen stimulation, and nitric oxide production, all of which are important immune responses. However, since tumour growth was not significantly affected by long-chain n-3 fatty acid supplementation in a low P/S diet, the potential importance of diet-associated changes in the measured immune variables is not clear. Nonetheless, the ability of dietary long-chain n-3 fatty acids to enhance the immune system should not be overlooked, since improved host immunity may be beneficial in respects other than primary tumour growth inhibition.

Our findings demonstrated that the polyunsaturated fat content (P/S ratio) of the diet significantly influences the effect of long-chain n-3 fatty acids on immune parameters assayed in tumour-bearing rats. This finding has important implications for current dietary recommendations, since it is presently advised that the North American population increase consumption of polyunsaturated fat (with a decrease in saturated fat intake) to achieve a P/S ratio of 1 (American Diabetes Association 1999, Krauss et al. 1996, Health and Welfare Canada), which is the same ratio fed in our high P/S diet. Our results, in an animal model of breast cancer, suggest that a lower level of polyunsaturated fat than is currently recommended by several health agencies may be required to enhance the immune benefits of long-chain n-3 fatty acids. Although cancer agencies have established dietary guidelines to reduce cancer risk (World Cancer Research Fund 1997), they do not currently have a recommendation for dietary P/S ratio and our findings suggest that they should not readily adopt the high P/S recommendation put forth by other health agencies since long-chain n-3 fatty acids fed in a high P/S diet neither suppressed tumour growth nor improved immune function in tumour-bearing rats. However, as discussed above, dietary long-chain n-3 fatty acids fed in a high P/S diet did have immune benefits in a healthy rat model. Thus, for cancer prevention, long-chain n-3 fatty acid supplementation in a high P/S diet may be unproblematic. Nonetheless, our results do show that the cancer treatment case may not be analogously unproblematic and that more work is needed before endorsing the recommendation of a high P/S diet during cancer.

Our work also compared, for the first time, the relative efficacy of dietary Gln, Arg, and OKG to improve immune defense in tumour-bearing rats. While dietary supplementation with Gln and Arg had limited or no immune effects in tumour-bearing rats, OKG supplemented in the diet had a significant enhancing effect on the cytotoxic capacity of NK cells and macrophages, as well as the proportion of activated T cells and nitric oxide production (Chapter 8). Overall, in the absence of diet-induced (either long-chain n-3 fatty acids or OKG) differences in tumour growth, the potential significance of diet-associated changes in the measured antitumour immune parameters is not obvious. However, the ability of specific nutrients, such as long-chain n-3 fatty acids and OKG, to enhance the immune system may be important since improved host immunity may be beneficial in terms of prevention of disease recurrence, metastasis, or infectious complications, and may be important in host tolerance to immunosuppressive treatments such as chemotherapy. Future experiments should test these potential benefits of specific "immunonutrients" fed to tumour-bearing rats using different models to reflect different human cancer situations.

### Immune Cell Membrane Composition

It is well established that changes in dietary fat composition can induce significant alterations in the composition and function of membranes in a variety of tissues, including immune cells (Field et al. 2000) and tumour cells (Calviello et al 1998, Rose et al. 1995). Therefore, this was a focus of our investigation into how dietary long-chain n-3 fatty acids alter membrane-mediated immune defense and tumour growth. Our work confirmed previous studies showing that dietary fish oil substantially altered the levels of n-3 and n-6 fatty acids in the major membrane phospholipids PC, PE and PI in immune cells (Chapter 3, 4, and 6). Since many of the functional immune parameters we assessed involved *in vitro* mitogen stimulation for 48-72 h after removal of cells from animals fed different diets, we extended our study to examine if diet-induced changes in immune cell phospholipid composition were maintained in culture and with mitogen activation. In general, many of the immune cell membrane changes induced by feeding long-chain n-3 fatty acids were maintained in an activated state. Thus, we demonstrated that our *in vitro* system is a valid means of studying the role of dietary long-chain n-3 fatty acids in modulating membrane-mediated immune functions since diet-induced changes in membrane phospholipid composition are maintained in mitogen-activated cells. Our findings also raise an important concern regarding the initial composition of cells used to study immune functions *in vitro*, since we demonstrated that different initial membrane compositions of immune cells respond differently to culture and activation (Chapter 4). It is possible that different starting amounts of n-3 and n-6 fatty acids (reflective of dietary intake) in immune cell membranes prior to culture and activation could make comparisons between studies and interpretation of results difficult. Finally, we did not design experiments to directly correlate specific diet-induced changes in membrane phospholipids with functional changes in immune cells, such as T cell activation, cytokine production, and NK cell cytotoxicity. However, this is an area for future research since we showed that immune cell membrane changes induced by dietary long-chain n-3 fatty acids are maintained in an activated state. In particular, diet-induced changes in PI and the role of the PI signaling pathway in NK cell cytotoxicity as well as T cell activation and cytokine production require further investigation in both healthy and tumour-bearing rats.

We found that the role of dietary fat in modulating immune cell membrane lipid composition is complicated by other factors, such as exercise-training and the presence of a tumour. For example, exercise-training altered membrane composition of immune cells and this effect was influenced by dietary fat composition (Chapter 3). The presence of a mammary tumour also altered immune cell membrane composition and some of the tumour-induced effects on immune cell phospholipids differed according to dietary fat composition (Chapter 6). In particular, feeding long-chain n-3 fatty acids to tumour-bearing animals maintained an immune cell lipid environment similar to that in healthy animals. This may have relevance for diet-induced upregulation of tumour immunity during cancer, although this requires further study. Clearly, in studies involving membrane-mediated immune functions, such as T cell activation and NK cell cytotoxicity, the diet fed to the animal impacts on membrane composition and interacts with factors that could affect subsequent membrane-mediated functions. This should be considered even if dietary design is not of primary interest to the investigators since dietary factors could influence changes induced by other parameters being studied, and make comparison of results and interpretation of data problematic.

### Tumour Cell Membrane Composition

The composition of mammary tumour cell phospholipids was altered by dietary long-chain n-3 fatty acids, especially when supplemented in a low P/S diet as opposed to a high P/S diet (Chapter 5). Analogously, fatty acids (DHA and LA) supplemented in the culture media changed the fatty acid composition of tumour cell phospholipids in our *in vitro* breast cancer model (Chapter 7). *In vitro* studies of tumour cells cultured in fatty acid-supplemented media can provide direct information on tumour cell growth and corresponding molecular changes. The influence of fatty acids on tumour growth in these assays is directly on tumour cells rather than on the whole tumour or on some aspect of the whole animal host, such as the immune system. We used the MDA-MB-231 breast cancer cell line in an attempt to localize membrane-mediated events involved in the tumour inhibitory mechanisms of DHA incorporated into tumour cell membrane phospholipids. We demonstrated that inhibition of proliferation, rather than enhanced apoptosis, is the mechanism by which DHA exerts its antitumour effect in our breast cancer cell model. The growth inhibitory effect of DHA may be associated with DHA-induced modulation of the fatty acid composition of the major membrane phospholipids PC and PI which are involved in intracellular signaling events. Therefore, we examined the effect of DHA on several growth-related proteins downstream of membrane-mediated events. Specifically, we found that levels of the D-type cyclins and proliferation-related proteins (PCNA and PRK) were not affected by DHA incubation, while expression of the mitotic cyclin B1 was decreased and p53 and pRb tumour suppressor proteins were increased by DHA supplementation. Further work is needed to establish a precise relationship between DHA-induced changes in tumour cell membrane composition and downstream growth-related events in this breast cancer model.

## 9.3 LITERATURE CITED

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# 10. APPENDIX

## (A) Composition of Various Solutions

### (1) Krebs Ringer-N-2-Hydroxyethylpiperazine-N'-2-Ethanesulfonic Acid (HEPES) Buffer (KRH)

| Chemical | Buffer Concentration (mM) | Stock Concentration (M)[1] |
|---|---|---|
| NaCl | 130 | 5.2 |
| HEPES | 10 | 0.4 |
| KCL | 5.2 | 0.21 |
| $CaCl_2$ | 1.4 | 0.06 |
| $NaH_2PO_4$ | 1.0 | 0.04 |
| $MgSO_4$ | 1.4 | 0.056 |

[1] Add 25 mL of each chemical stock solution and complete with distilled water. Adjust pH of 7.4. Sterilize by membrane filtration and store at room temperature.

### (2) Splenocyte Lysis Buffer

| | |
|---|---|
| $NH_4Cl$ | 155 mM |
| EDTA | 0.1 mM |
| $KHCO_3$ | 10 mM |

Make above solution to the following concentrations with $ddH_2O$. Adjust pH to 7.4.

### (3) Phosphate Buffered Saline (PBS)

| | |
|---|---|
| NaCl | 8.00 g |
| KCl | 0.20 g |
| $Na_2HPO_4$ | 1.15 g |
| $KH_2PO_4$ | 0.20 g |

Add these chemicals to 1 L of distilled water. Adjust pH to 7.4.

**(4) Hanks Balanced Salt Solution**

| Chemical | Final Concentration (g/L) |
|---|---|
| KCl | 0.40 |
| $KH_2PO_4$ | 0.10 |
| NaCl | 8.0 |
| $NaH_2CO_2$ | 1.27 |
| Glucose | 1.00 |

To make 1 L: Use 25 mL of each chemical stock solution and add 1 g glucose. Make to 1 L with $ddH_20$, pH to 7.4. Sterilize by membrane filtration.

**(5) Tris-Buffer Saline (TBS)**

2.42 g Tris-base (20 mM)

8 g NaCl (137 mM)

Dilute to 1 L with $ddH_2O$, adjust pH to 7.6 with HCl (3.8 mL 1M HCl)

**(6) TBS-Tween (0.1%)**

Dilute 1 mL Tween-20 in 1L of TBS

**(7) Gel Running Buffer**

| | |
|---|---|
| 25 mM Tris Base | 3.02 g |
| 192 mM Glycine | 14.4 g |
| 0.1% SDS | 1.0 g |
| $ddH_2O$ | 1 L |

**(8) Western Blot Transfer Buffer**

| | |
|---|---|
| 25 mM Tris Base | 3.02 g |
| 192 mM Glycine | 14.4 g |
| 20% Methanol | 200 mL |
| $ddH_2O$ | 800 mL |

## (B) Solutions for Cytokine ELISA

### (1) IL-2

**Binding Solution:** 0.1M $Na_2HPO_4$, adjust pH to 9.0 with HCl.

**PBS Solution:** 80.0 g NaCl, 11.6 g $Na_2HPO_4$, 2.0 g $KH_2PO_4$, 2.0 g KCl, q.s. to 10 L, pH to 7.0.

**PBS/Tween:** Add 0.5 mL of Tween-20 to 1 L PBS.

**Blocking Buffer:** Add FCS (100 g/L) to PBS. Filter before use.

**Blocking Buffer/Tween:** Add 0.5 mL Tween-20 to 1 L Blocking Buffer.

**ABTS Substrate Solution:** Add 150 mg 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) to 500 mL of 0.1 M anhydrous citric acid in $ddH_2O$, pH to 4.35 with NaOH. Aliquot 11 mL per vial and store at -20 °C. Add 10 μL 30% $H_2O_2$ prior to use.

**30% $H_2O_2$:** Store in aliquots at –20 °C.

**Standard concentrations (pg/mL):** 2000, 1500, 1000, 750, 500, 250, 125, 50, 25, 15.

### (2) IFN-γ

**Coating Buffer:** Dulbecco's Phosphate Buffered Saline (DPBS), pH to 7.4.

**Blocking Buffer:** DPBS with BSA (10g/L), 0.25% Tween-20 and heat inactivated FCS (250 g/L).

**Wash Buffer:** DPBS with 0.05% Tween-20.

**Substrate:** Tetramethylbenzidine (TMB) and hydrogen peroxide (Sigma One-Step, Product No. T8540).

**Stop Solution:** 1 M $H_2SO_4$

**Standard concentrations (pg/mL):** 1620, 540, 180, 60, 20.

### (3) TNF-α

**PBS:** 80.0 g NaCl, 11.5 g $Na_2HPO_4$, 2.0 g $KH_2PO_4$, 2.0 g KCl, q.s. to 10 L with $ddH_2O$, pH to 7.4.

**Wash Buffer:** 0.05% Tween-20 in PBS, pH to 7.4.

**Diluent:** BSA (1 g/L), 0.05% Tween-20 in Tris-buffered saline, pH to 7.3.

**ABTS Substrate Solution:** Add 150 mg 2,2'-azino-bis-(3-ethylbenzthiazoline-6-sulfonic acid) to 500 mL of 0.1 M anhydrous citric acid in $ddH_2O$, pH to 4.35 with NaOH. Aliquot 11 mL per vial and store at -20 °C. Add 10 μL 30% $H_2O_2$ prior to use.

**30% $H_2O_2$:** Store in aliquots at –20 °C.

**Standard concentrations (pg/mL):** 2000, 1500, 1000, 750, 500, 250, 125, 100, 50, 30, 15.

## (C) Premlinary *In Vitro* Work for Chapter 7

Preliminary experiments using 60 µM LA, 60 µM DHA, 60 µM LA + 60 µM DHA, and 120 µM LA are shown in **Figure C-1**. There were no differences between fatty acid treatments at these concentrations.

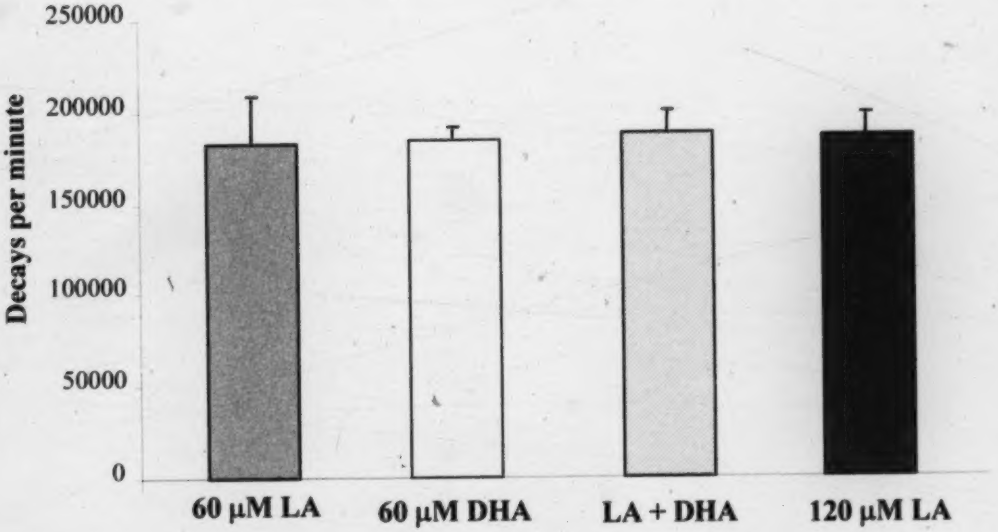

**FIGURE C-1. Proliferation of MDA-MB-231 Breast Cancer Cells After 72 h Incubation With Fatty Acids.** Cells were seeded (2 x $10^4$ cell/mL/well) in 24-well sterile, flat-bottom tissue culture plates in culture media containing fetal calf serum (50 g/L) and allowed to adhere. After 48 h, the standard cell culture media was replaced with fresh media containing fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid) and cells were further incubated for 72 h. At 4 h prior to harvesting, cells were pulsed with [methyl-$^3$H]-thymidine as described in Materials and Methods in Chapter 7. Bars represent means ± SEM (*n* = 2/treatment).

Preliminary experiments using 90 μM LA, 90 μM DHA, 90 μM LA + 90 μM DHA, and 180 μM LA are shown in **Figure C-2**. Thymidine incorporation by MDA-MB-231 breast cancer cells was decreased when cells were cultured for 72 h with 90 μM DHA compared with cells incubated with 90 μM LA. In addition, incubation of cells with 90 μM DHA in the presence of 90 μM LA (LA + DHA) also resulted in decreased [$^3$H]-thymidine uptake by tumour cells. Since we were concerned that higher fatty acid concentrations added to the media would be toxic to cells, we had to ensure that supplementation of 90 μM LA + 90 μM DHA did not inhibit cell proliferation due to a fatty acid-induced cytotoxic effect. Thus, a control was used (180 μM LA) which contained an equal total fatty acid concentration to the LA plus DHA treatment group. Our results from these preliminary experiments show that the growth inhibitory effect of LA + DHA at these concentrations (Figure C-2) may be due to the total amount of fatty acid in the media (180 μM) since [$^3$H]-thymidine uptake by MDA-MB-231 cells was similar for cells cultured with 180 μM LA and 90 μM LA + 90 μM DHA and both were lower than cells cultured with the lower level of LA (90 μM).

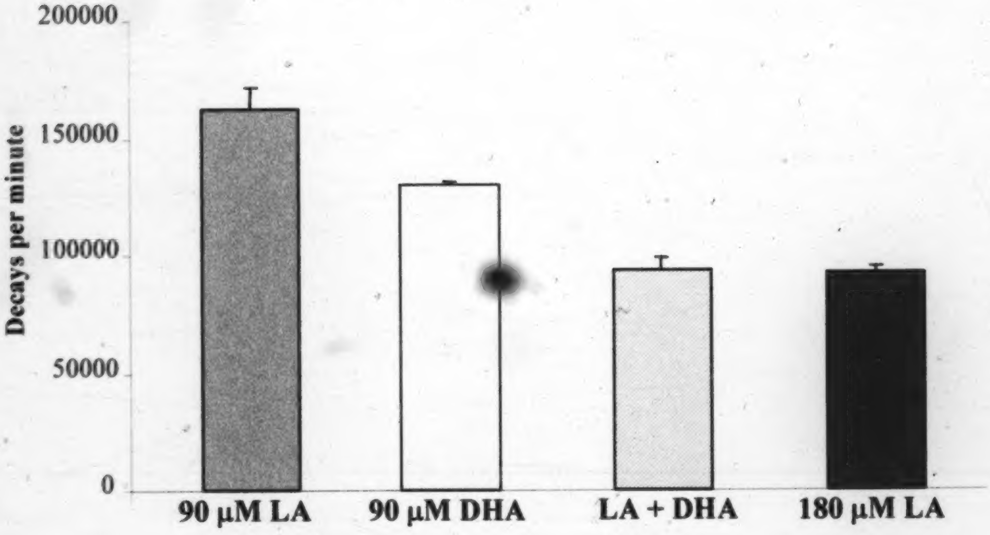

**FIGURE C-2. Proliferation of MDA-MB-231 Breast Cancer Cells After 72 h Incubation With Fatty Acids.** Cells were seeded (2 x $10^4$ cell/mL/well) in 24-well sterile, flat-bottom tissue culture plates in culture media containing fetal calf serum (50 g/L) and allowed to adhere. After 48 h, the standard cell culture media was replaced with fresh media containing fatty acids (LA, linoleic acid; DHA, docosahexaenoic acid) and cells were further incubated for 72 h. At 4 h prior to harvesting, cells were pulsed with [methyl-$^3$H]-thymidine as described in Materials and Methods in Chapter 7. Bars represent means ± SEM (*n* = 2/treatment).

Based on these preliminary experiments, we chose LA and DHA concentrations in between the two sets of concentrations (60 µM and 90 µM, Figures C-1 and C-2). Thus, the fatty acid concentrations chosen for the experiments described in Chapter 7 were 75 µM LA, 75 µM DHA, 75 µM LA + 75 µM DHA, and 150 µM LA.